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THE SWIMMING OF LEPTOSYNAPTA

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The fact that young adult leptosynaptids swim, or pass through a post-larval swimming stage, has not been adequately recorded, and is not generally recognized by the students of the Holothuria. Adult leptosynaptids are usually considered to be exclusively bottom-dwelling forms, and the only reference to the contrary that I have found is a brief and incomplete statement by Clark (1907) to the effect that: "Few, if any, synaptids swim, except in the larval state, but young ones, up to 2 cm. in length, are sometimes found floating, *if not swimming*, in the water." The italics are mine. I am therefore placing on record the following observations on the swimming habits of these animals, made during the summers of 1944 and 1945 at Woods Hole, Mass. I observed the same phenomenon a few years earlier (in 1939), but no detailed records were made at that time.

On August 14th, 1944, between 9:15 and 9:30 P.M., while collecting the swarming heteronereis form of *Nereis limbata* in Eel Pond, ten small, colorless, transparent holothurians were observed swimming more or less aimlessly near the surface of the water. They were being carried partly by their own movements and partly by the tidal currents, across the field of light cast on the water by the 100-watt bulb of the Nereis collecting lamp. All ten were collected. These swimming animals were 30 to 50 mm. in length and about 3 mm. in diameter. When touched in the water by the cheesecloth surface of the Nereis collecting net, they ceased their swimming movements and contracted, shrinking to about one-fourth of their extended length by the expulsion of fluid, became opaque, and sank slowly toward the bottom. If collected by being dipped up with a quantity of sea water in a finger bowl without being disturbed, they continued their swimming movements in the dish. Jarring of the dish caused extrusion of fluid, contraction, and sinking of the animal. The change from the glass-like transparency of the extended form to the white opacity of the contracted form is extremely striking.

The animal swims only when in the extended state. The effective stroke occurs after the animal has assumed the shape of a wide U (see Fig. 1). The longitudinal muscles on one side of the body suddenly contract, drawing the posterior end of the body up to and past the tentacled anterior end, and then, as suddenly, relax. Relaxation results in the resumption of the U-shaped form, and is followed by another contraction of the longitudinal muscles. A series of these contractions creates an effective swimming movement, comparable to the breast-stroke of human swimmers,

in the direction of the base of the U. If, on one stroke, the head crosses above the posterior region, on the next it usually crosses below, so that there is an alternation of movements, presumably brought about by the contraction of sets of longitudinal muscles of opposite sides of the body. At the same time, the whole body turns frequently, so that locomotion is not in one direction, but is more or less aimless. Nevertheless, the swimming leptosynaptids can move across a ten-inch finger bowl in the course of four or five minutes.

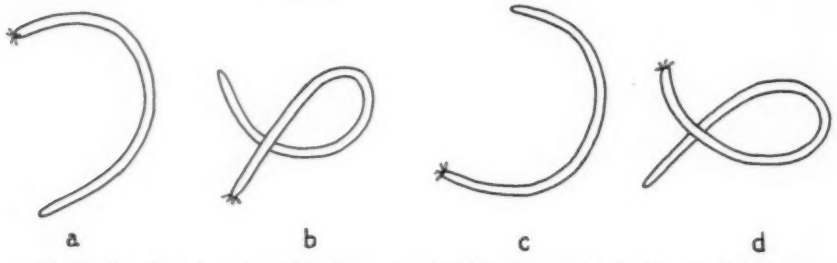

FIGURE 1, a, b, c, d. Successive stages of swimming movements of *Leptosynapta inhaerens*. The effective stroke, directed toward the base of the U, occurs between a and b, and between c and d. During the period between b and c, there has occurred a rotation of the entire body, on the axis of the U, through 180 degrees.

On the following evening (August 15) nine more leptosynaptids were observed swimming in Eel Pond between 9:25 and 10:00 P.M., and were collected. At 10:00 P.M., on entering the darkened laboratory, I observed that four of the leptosynaptids collected the previous evening (and left in a bowl of standing sea water on the sea water table since that time) were in the extended condition and undergoing rapid swimming movements. These animals had remained in the contracted state at the bottom of the dish during the day (see Fig. 2). Similar observations were made for these and other animals on subsequent evenings. Swimming is undoubtedly a

FIGURE 2. Photograph of five *Leptosynapta inhaerens*, living young adult "swimming forms." Four are in the completely contracted, opaque state; the fifth almost completely contracted, and partially transparent. Centimeter scale indicated below.

dark-conditioned phenomenon, yet placing leptosynaptids in the darkroom during the day did not induce swimming. Presumably either a certain period of illumination or of complete rest is first necessary. Quatrefages (1842) early called attention to the greater general activity of Leptosynapta at night as compared with that during the day. On the morning of August 16th, at 11:00 A.M., one leptosynaptid in a large finger bowl (covered with cheesecloth, over which a slow stream of sea water was flowing) was still swimming, but did not show the alternate criss-crossing of the two ends as observed in nature. This is the only Leptosynapta observed swimming during the day.

A complete record of Eel Pond collections is as follows:

Date	Time	Number collected
Aug. 14, 1944	9:15– 9:30 P.M.	10
15	9:25–10:00	9
16		2
17	9:10–9:40	5
18		none
22	10:00 P.M.	1
23	9:30 P.M.	2
28		none
29		"
30		"
31		"
Sept. 4		"
6		"
7		"
	1944 Total	29
Aug. 25, 1945	10:30 P.M.	1
Sept. 6	9:00 P.M.	2
7		1
8		1
9	8:45 P.M.	1
	1945 Total	6

The 1945 specimens were collected by Miss Catherine Henley and Miss Roberta Lovelace. Nereis collections were made almost every night from early June until the end of September, 1945, but no leptosynaptids were seen other than the six listed above. The last specimens taken in 1944 were larger than those first collected. Those kept for two or three weeks in the laboratory grew slightly and showed an increase in number of tentacular sensory cups. These facts indicate the probability that Leptosynapta swims only during a limited period of its young adulthood.

An examination of the collected specimens revealed only a few grains of sand, and some diatoms and other debris in the intestine. Presumably a necessary condition for swimming is absence of intestinal sand which would prevent floating. When the animals swell to the extended condition, the intestine is not filled with water. The intestine can be seen within the body and is approximately of normal diameter. The water probably enters the perivisceral cavity. Tests were made to ascertain the places of exit of the fluid from the body by dropping extended animals into a suspension of Chinese ink in sea water. These tests were somewhat incon-

clusive, but indicated a general release of fluid along the major portion of the body surface, rather than any markedly localized release.

A preliminary examination of the anchors, anchor plates, and other spicules indicated size characteristics somewhat intermediate between those of the two common Woods Hole species, *Leptosynapta inhaerens* and *Leptosynapta roseola*. The radial plates of the calcareous ring were seen to be perforated. Some of the material was then sent to Dr. Elisabeth Deichmann, who kindly identified the swimming forms as *Leptosynapta inhaerens* (O. F. Müller). This species, as found in the Woods Hole region, was described by Clark (1899). The deviations from the typical adult characteristics which were found in the swimming forms were deviations characteristic of young individuals.

Leptosynaptas have apparently never before been seen swimming in Eel Pond, even by such a veteran collector as Mr. George M. Gray. Fish (1925), who studied the extensive plankton collections of the Woods Hole Fisheries Laboratories, records the presence of only one leptosynaptid—as follows: "A specimen of *Leptosynapta inhaerens* (Müller), 20 mm. long, was taken on Sept. 19 after a hard northeast storm. This was not a free-swimming form and would not normally occur in surface collections." Dr. George L. Clarke, of the Woods Hole Oceanographic Institution (personal communication), has never taken a holothurian in his plankton hauls.

Swimming of certain other holothurians is not unknown. There is a pelagic form, *Pelagothuria natatrix*, from the Eastern Pacific, described by Ludwig (1893). For *Stichopus natans*, swimming movements were observed by Sars (quoted by Ludwig, 1889–92). Prior to the present work these constituted the rare exceptions to the general rule that adult holothurians do not swim.

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CORRELATION BETWEEN THE POSSESSION OF A CHITINOUS CUTICLE AND SENSITIVITY TO DDT *

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Introduction

The remarkable insecticidal qualities of 2, 2-bis (p-chlorophenyl) 1, 1, 1, trichloroethane, more commonly called "dichloro-diphenyl-trichloroethane" or just "DDT," were discovered by Paul Müller of the J. R. Geigy Company of Switzerland a little more than five years ago (Lauger, Martin, and Müller, 1944). The first samples reached this country in August, 1942. Researches in Switzerland, Germany, England, and the United States, accelerated by war-time urgency, have developed a voluminous amount of data. Most of these data are purely practical and will not be reviewed here; [2] further, these economic reports and papers are appearing so rapidly that it is very difficult to separate fact from fancy in the confusing maze of seeming contradictions. Certainly numerous popular articles and stories are dangerously misleading (see, e.g., warning by Cox, 1945, and Anonymous, 1945). The fact remains, however, that DDT is a remarkably potent insecticide and that from the human point of view it can be used with relative safety for certain insect pests (flies, mosquitoes, lice, bedbugs, etc.).

Since this new compound is so extremely toxic to many insects, one is naturally led to inquire what its effects are on other animals. Both a desire to find more favorable animals for studying the physiological effects of DDT, and a need to evaluate the effects of the compound on the entire biota of natural waters, led to determinations of the toxicity of DDT to representatives of various animal phyla. During the course of making these determinations it was noted that animals with exoskeletons, especially chitinous exoskeletons, were notably susceptible. Experiments were accordingly performed to test the validity of this correlation. From these experiments it was concluded that the correlation is valid. A hypothesis is developed in this paper that chitinous cuticles selectively concentrate DDT from the bathing media by adsorption and result in a higher concentration of the toxin inside the animal. There is good evidence to indicate that the relation of the chitinous cuticle to the selective action of DDT is no more than that of a concentrating mechanism. The actual lethal action of DDT seems to be another problem (see, e.g., Yeager and Munson, 1945).

* Paper No. 2267, Scientific Journal Series, Minnesota Agricultural Experiment Station, St. Paul 8, Minnesota.

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[2] Large numbers of papers can be found, especially in the February, 1944 and June, 1945 issues of the *Journal of Economic Entomology*. The review in *Soap* is a useful digest (Anonymous, 1945).

The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and the University of Pennsylvania, and, later, the University of Minnesota. Most of the data were incorporated in a paper presented at the meetings of the Entomological Society of America in New York City, December 14, 1944.

Materials and Methods

For the purpose of this study only aquatic species or aquatic stages were employed. The toxin was applied as what seems to be a collodial suspension in the bathing medium because the solubility of DDT in water is very low, at room temperatures too low to show significant toxicity to susceptible animals. Mechanically shaking DDT in distilled water (68 hours), filtering, and then centrifuging with an angle centrifuge to remove minute particles (15,000 G for one hour),[3] gave a solution that slowly killed about 50 per cent of the mosquito larvae tested at 15° C. but had no significant effect on larvae at 28° C. Several slightly different tests gave similar results. Comparing these data with data from bio-assay of known dilutions of collodial suspensions prepared from acetone solutions, we concluded that the solubility of DDT in water probably lies between 0.2 and 1.0 parts per billion when prepared by agitation in water.[4] Presumably suspensions from acetone solutions would give much higher solubility values, perhaps one hundred times as high (Lewis and Randall, 1923). The exact physical state, then, of the preparations we are calling "suspensions" is uncertain but at least the preparations are sufficiently homogeneous to dilute satisfactorily.

The suspensions were prepared by adding a known volume of an acetone solution of DDT beneath the surface of a known volume of water (distilled, fresh, or sea water). A suspension of one part per million that will give reproducible results and dilute satisfactorily can be prepared by adding one ml. of a 0.1 per cent DDT solution beneath the surface of a liter of water. Stronger suspensions were usually made from more concentrated DDT solutions. Suspensions as strong as one part per hundred thousand are not stable and do not dilute satisfactorily.

Suspensions of one part per million in sea water diluted with distilled water and tested by bio-assay with mosquito larvae indicated that the suspensions in sea water have essentially the same characteristics as those in fresh and distilled water.

Since all suspensions contained a small amount of acetone, at least two controls were always used. One was a control in the water indicated; the other, the same plus whatever was the highest per cent of acetone in any suspension. In general, most animals were indifferent to 0.1 per cent acetone. The few cases where 0.1 per cent acetone seemed to have a possibly deleterious effect (e.g., certain Coelenterata), tests were repeated with lower acetone concentrations. The more dilute suspensions, prepared by dilution of a one part per million suspension containing 0.1 per cent acetone, had only negligible amounts of acetone.

[3] This is a force sufficient to sediment bacteriophage particles of approximately 20 mμ diameter but should not affect molecules several times larger than the molecule of DDT.

[4] This is very much lower than the one part per million figure given by Neal *et al.* (1944). Our bio-assay data, as well as similar data from other laboratories, are not consistent with this high estimate.

The amount of fluid used varied with the size of the animals. Protozoa and certain other forms were tested in watch glasses containing 10 ml. of fluid. Other forms were tested in shell vials or finger bowls containing 25–200 ml. of fluid. Of course, single series and replicates were with uniform volumes. Continuously flowing suspensions were not feasible; the more difficultly cultured marine animals were changed to fresh suspensions twice daily. For species that did not require frequent changing of the medium, subsequent assays with mosquito larvae or hermit crabs were commonly employed to verify the continued toxicity of test fluids.

Specimens were obtained from various sources. Protozoa were from laboratory cultures and were identified by Dr. D. H. Wenrich. Marine forms were collected at Woods Hole, mostly by the Supply Department, and the names used are those current at that laboratory. Other forms were collected or purchased, and identified by the authors.

Chemical tests for the presence of chitin were routinely performed on exoskeletons using the methods given by Campbell (1929). Positive results were obtained with Arthropods and the perisarc of Colenterates. The Bryozoan used (*Bugula*) gave atypical results suggesting a skeleton composed of something similar to, but not, chitin.[5]

For adsorption tests cuticle was removed from Horseshoe Crabs (*Limulus polyphemus*), manually cleaned of cells, rinsed thoroughly with distilled water, and dried. Part of this material was treated with 5 per cent NaOH at 100° C. until the color was removed and the pieces were negative to protein tests. This latter material is referred to herein as "chitin" although tests showed that a small percentage of the material had been changed to chitosan (see Campbell, 1929). Smaller amounts of cuticle were prepared from cockroaches and used in a few experiments, but most of the adsorption work was done with the more available cuticle of *Limulus*.

All tests were performed in replicate. Many of them were repeated.

Results

The results obtained are summarized in the accompanying table. In all cases the toxin (DDT) was applied as a colloidal suspension in the bathing medium. The resistances or susceptibilities shown refer only to the indicated concentration in the surrounding medium. The figures are not comparable to median lethal doses and should not be interpreted as implying that "resistant" animals would be resistant to the injection of solutions or emulsions.

[5] The cuticle of *Bugula* is variously referred to in the literature as "chitinous" or "material akin to chitin." We applied the various chemical tests used by Campbell (1929) and others. The cuticle is not dissolved in hot concentrated KOH. After prolonged heating (which changed control pieces of known chitin to chitosan completely) it only crinkled in 3 per cent acetic acid instead of dissolving. The addition of one per cent H_2SO_4 gave no change and no precipitate. Another piece treated with I + KI in water turned brown but only very slowly. After the addition of one per cent H_2SO_4 it remained brown for several minutes and then after 5–10 minutes slowly changed to a *greenish-brown* (instead of the clear violet given by chitosan). It dissolved immediately in 75 per cent H_2SO_4 but after standing no crystals resembling chitosan sulfate crystals were found. These tests suggest a similarity to chitin but certainly, at least in this species, the bryozoan cuticle is not true chitin.

TABLE I

The effective toxic concentrations of DDT for various animal phyla

All tests were replicated at least once. Susceptible species were tested repeatedly. Except where indicated otherwise tests were continued for 7 days or longer. The fluid on species that do not survive well in stagnant water was renewed daily or more frequently.

Group	Species	Temperature, C.	Type of water	Concentrations of DDT suspension					
				1:1,000,000,000	1:100,000,000	1:10,000,000	1:1,000,000	1:100,000	1:10,000
Protozoa	*Ameba proteus*	room	fresh	——	——	——	No effect (14 da)	Slow effect?	Slowly killed (3–10 da.)
	Paramecium sp.	room	fresh	——	——	No effect	No effect	No effect (1 mo.)	——
	Stylonychia putrina	room	fresh	——	——	——	No effect	——	No effect (1 mo.)
	Euglena gracilis	room	fresh	——	——	——	No effect	——	No effect (1 mo.)
	Chilomonas sp.	room	fresh	——	——	——	No effect	——	——
	Peranema sp.	room	fresh	——	——	——	No effect	——	No effect (1 mo.)
Coelenterata	*Obelia* sp.[1]	15°	sea	——	Many killed	Killed (1–2 da.)	Killed (<1 da.)	——	——
	Campanularia sp.[1]	15°	sea	——	No effect	Affected slightly[2]	Affected[2]	——	——
	Tubularia sp.	15°	sea	——	No effect	Killed slowly	Killed	Killed	——
	Pennaria sp.	15°	sea	——	No effect	No effect	No effect	Affected[3]	——
	Hydractinia sp.	15°	sea	——	No effect	No effect	No effect	Affected[3]	——
	Hydra fusca	15°	fresh	——	——	No effect	No effect	Affected[3]	——
	Astrangia sp.	15°	sea	——	——	——	——	——	——
Platyhelminthes	*Phagocata* sp.	room	fresh	——	——	No effect (1 mo.)	Killed (7–40 da.)	Killed (7–20 da.)	Killed (1–6 da.)
Nematoda	*Anguillula aceti*	room	fresh	——	No effect	No effect	No effect	No effect	No effect (1 mo.)
	Ascaris lumbricoides	35°	alkaline saline	——	——	——	No effect	No effect	No effect
Rotifera	*Philodina* sp.	room	fresh	——	No effect	No effect	No effect	No effect (1 mo.)	No effect
	Diplax sp.	room	fresh	——	——	——	No effect	No effect (1 mo.)	No effect
	Monostyla sp.	room	fresh	——	——	——	Some killed slowly (1–2 mo.)	Killed slowly (10–30 da.)	Killed slowly (7–10 da.)
Gastrotricha	*Chaetonotus* sp.[4]	room	fresh	——	——	No effect	No effect	——	——
Bryozoa	*Bugula* sp.	15°	sea	——	No effect (10 da.)	Killed slowly (4–6 da.)	Killed slowly (3–4 da.)	——	——

[1] At least two, probably three, species of *Obelia* and *Campanularia* were used in these tests but identification of species in this group is quite difficult.

[2] *Tubularia* stems selected by Dr. L. G. Barth were set-up as in regeneration studies. All specimens regenerated but polyps quickly died in stronger concentrations and almost without exception did not regenerate a second time. Controls regenerated repeatedly.

[3] At 1:100,000 the specimens of *Hydra*, *Hydractinia*, and *Astrangia* were rounded and slow in reacting at the end of six days but were still living.

[4] These gastrotrichs were not tested separately. They were present in one of the rotifer cultures, and data cover only those test dishes, in which they were found.

TABLE I—*Continued*

Group	Species	Temperature, C.	Type of water	Concentrations of DDT suspension					
				1:1,000,000,000	1:100,000,000	1:10,000,000	1:1,000,000	1:100,000	1:10,000
Mollusca	*Littorina* sp.	room	sea	——	No effect	No effect	No effect	No effect	Killed slowly (6–7 da.)
	Mytilus sp.[5]	15°	sea	——	——	——	No effect	——	——
	Helisoma trivolva	25°	fresh	——	——	——	——	No effect	No effect
	Physa sp.	25°	fresh	——	——	——	——	No effect	Killed slowly
Echinodermata	*Thyone* sp.	room	sea	——	——	No effect	No effect	No effect	No effect
	Asterias sp.	room	sea	——	——	No effect	No effect	Killed (4 da.)	Killed (2 da.)
Annelida	*Aeolosoma* sp.	room	fresh	——	——	No effect	No effect?	Effect?[6]	——
	Polydora sp.[5]	15°	sea	——	——	——	——	No effect (7 da.)	——
	Nereis virens	15°	sea	——	No effect	No effect	No effect	Killed (4 da.)	Killed (2–3 da.)
	Lumbricus terrestris	15°	fresh[7]	——	——	——	——	No effect	No effect
Crustacea	*Artemia salina*	15°	sea	Most killed 2–6 da.	Killed (2–6 da.)	Killed (1–5 da.)	Killed (1–5 da.)	Killed (2–5 da.)	Killed (1–5 da.)
	Gammarus sp.	15°	fresh	Most killed (1–6 da.)	Killed (1–3 da.)	Killed (1–3 da.)	——	——	——
		28°	fresh	<half-killed (6 da.)	Killed (1–3 da.)	Killed (1–2 da.)	Killed (1 da.)	Killed (<1 da.)	Killed (<1 da.)
	Daphnia sp.	15°	fresh	Effect?[8]	Most killed (2–7 da.)	Killed (1–5 da.)	——	——	——
	Cyclops sp.	room	fresh	——	No effect	No effect?	Killed (<3 da.)	Killed (<3 da.)	Killed (<3 da.)
	Pagurus sp.	15°	sea	——	——	Killed (1–3 da.)	Killed (1 da.)	——	——
	Emerita talpoida	15°	sea	——	——	Effect?	Killed (1–2 da.)	Killed (1–2 da.)	——
Insecta	*Aedes aegypti* larvae	15°	fresh	Half killed (7 da.)	Killed (2–7 da.)	Killed (2–4 da.)	Killed (2–4 da.)	——	——
		28°	fresh	No effect (7 da.)	Most killed (7 da.)	Killed (1–4 da.)	Killed (1–2 da.)	——	——
	Culex pipiens larvae	15°	fresh	——	——	Killed (<1 da.)	——	——	——
		30°	fresh	——	——	Killed (1–3 da.)	——	——	——
	Chironomus sp. larvae	room	fresh	——	——	Killed (1–2 da.)	——	——	——
	Chaoborus sp. larvae[9]	10°	fresh	Some killed (5 da.)	Killed (5 da.)	Killed	——	——	——
		30°	fresh	No effect (5 da.)	Most killed (5 da.)	Killed	——	——	——
Fungi	*Rhizopus nigricans*	room	fresh	——	——	——	——	——	No effect
	Penicillium sp.	room	fresh	——	——	——	——	——	No effect
	Saccharomyces cervisiae	room	fresh	——	——	——	No effect	No effect	——

[5] Only a few specimens (not a statistically significant number) were tested of *Mytilus* and *Polydora*. The negative results suggest that these are both resistant species, as are other members of their phyla.

[6] At 1:100,000 there was an initial decrease in numbers followed by an increase. It would seem that some are killed but that the survivors reproduce readily.

[7] The earthworms were tested in flat dishes with the fluid not sufficiently deep to cover the animals completely.

[8] Anderson (1945) records an effect on *Daphnia* at concentrations greater than one part per billion and no effect at concentrations lower than one part per billion. Apparently his cultures were slightly more susceptible to DDT than ours.

[9] Experiments on *Chaoborus* by Dr. Hsing Yun Fan.

Of the species tested, the Protozoa, Nematoda, Gastrotricha, Rotifera, Mollusca, Annelida, and Echinodermata were resistant to DDT suspensions or in the case of a few species, were slowly killed by very concentrated suspensions. The single species of the Platyhelminthes tested was comparatively resistant. The single species of Bryozoa was quite susceptible. And, the various species of Arthropoda and those Coelenterata with a complete perisarc were highly susceptible. The most interesting data come from the hydrozoan Coelenterata where those species with a complete chitinous perisarc (*Obelia* and *Campanularia*) are highly susceptible, those species with a chitinous perisarc over only the main stalk (*Tubularia* and *Pennaria*) were less susceptible, and those without a perisarc (*Hydra* and *Hydractinia*) were nearly resistant. The bryozoan, *Bugula*, is an intermediate which is almost as susceptible to DDT as the arthropods and sensitive colenterates. *Bugula* has a complete cuticle which is composed of some substance seemingly similar to but not identical with chitin.[5] Correlated with this similarity is the more or less similar relationship to DDT poisoning from dilute solutions. The most reasonable assumption at present is that the cuticle of *Bugula* behaves towards DDT similarly to chitinous cuticles but that it is less effective (or possibly the animals are really more resistant).

The data presented herein do not include vertebrates. The most nearly comparable data on fishes indicate an intermediate sensitivity (Eide, Deonier, and Burrell, 1945; Ginsburg, 1945) and that concentrations entirely adequate for mosquito control (i.e., well above the minimum lethal concentration) do not injure fish (Metcalf *et al.*, 1945). With terrestrial vertebrates truly comparable data are not available. The closest parallel is with aerosols and mists; very high concentrations of DDT applied in this manner cause little or no injury to mammals (Neal *et al.*, 1944). DDT in solution in oil can be absorbed through mammalian skin (Draize *et al.*, 1944) but the dosage needed to produce an effect is fairly high. Also, it is well known that DDT has been approved for use as a powder on vertebrates to kill ectoparasitic insects. The data, then, suggest that for external applications more or less comparable to our data, fish occupy an intermediate position in susceptibility—being some ten to one hundred times more resistant than mosquito larvae. Mammals seem to be much more resistant to external applications than either fish or insects.

The obvious correlation of the above data is to the chitinous exoskeleton. Actually there are a number of possibilities, notably (1) that some of the animal groups are truly more or even completely resistant to the toxin; (2) that certain animal groups may be able to detoxify or excrete DDT better than others; (3) that there may be a reaction between a chitinous cuticle and DDT to produce some other more toxic substance; or (4) that the susceptibility may be due solely to penetration and accumulation which is favored by a chitinous cuticle. The evidence at hand suggests that both the first and last named possibilities are partly correct. Considering the points in order:

Data derived from the feeding and injection of DDT solutions (not suspensions) and emulsions show that on a dosage/weight basis mammals have approximately the same order of susceptibility as cockroaches (Draize, *et al.*, 1944; Smith and Stohlman, 1945; Chadwick, 1945). While some variability is obtained among insects (Chadwick, 1945), the fact remains that internal applications do not

show the tremendous differences between vertebrates and insects that external applications show.

However, there must be a considerable amount of variability in true resistance among different groups on invertebrates. *Ascaris* is immune to external applications of DDT suspensions but the median lethal dose for injection of emulsions (on a dosage/weight basis) seems at least several times as high as the MLD for cockroaches.[6] And, injection of DDT emulsions into the common aquarium snail, *Helisoma trivolva,* was apparently without effect even when one mg. of DDT was injected into a snail whose weight (without the shell) was less than a gram. Unfortunately, we have no precise knowledge of the site of injection but at least it was into the interior of the animal and sometimes into the tissues. It seems clear from these data (1) that there must be considerable difference among various animals as to the effect of a given amount of DDT after it is *in* the animal, and (2) that relative absorbability also varies.

The second possibility, detoxification or excretion by certain groups, seems unlikely both because of the similarity of vertebrate and insectan median lethal doses on injection and because of the great dissimilitary in excretory organs. The role of detoxification may actually be considerable. One non-toxic breakdown product, dichlorodiphenylacetic acid, has already been positively identified in mammalian urine (White and Sweeney, 1945). Detoxification, however, does not seem to contain the full answer to the wide variations in susceptibility reported in this paper.

The third possibility, a new and more potent toxin arising from a reaction with chitinous cuticles, seems eliminated by several points. The above mentioned similarity of median lethal doses and symptoms following injection of emulsions is against it. Also pertinent is the fact that DDT suspensions have no visible effect on cultured tissues whether chitin particles are added to the medium or not (Lewis and Richards, 1945).

The above considerations led to two different types of tests on the fourth possibility; namely, that cuticles might facilitate the entry of DDT into animals. If chitinous cuticles facilitate the entry of DDT it might be possible to find aquatic invertebrates which are immune to external applications but killed by injections. We have data on one such case. *Acaris* does not have a chitinous cuticle and is completely resistant to immersion in the strongest DDT suspensions. It is killed by the injection of emulsions although requiring a somewhat higher dose than cockroaches and vertebrates.

While these data on *Ascaris* are suggestive, they, of course, do not prove anything about chitinous cuticles. More direct data were sought by trying to find a possible mechanism by means of which chitinous cuticles might facilitate the entry of DDT into an arthropod or coelenterate. The following data suggest that such a mechanism does, indeed, exist and that adsorption by chitin plays an important role in it.

Obviously, DDT can penetrate insect cuticles since external applications can kill even when the possibility of ingestion is eliminated. Tests showed that DDT could be adsorbed from water by activated charcoal. DDT suspensions in water

[6] We determined the median lethal dose for *Ascaris* only to a rough order of magnitude and so prefer not to cite a definite quantitative figure.

lost all toxicity to mosquito larvae after being stirred with charcoal and filtered. It follows from this that the DDT particles have been removed by the charcoal. Similarly, we found that DDT could be adsorbed from aqueous suspensions by arthropod cuticle and even better by purified chitin. The results are only qualitative; accordingly, tabular data are not presented. Cuticle and purified chitin from *Limulus* and from cockroaches were prepared as described in the section on methods. An excess of either material was added to a moderately dilute suspension of DDT in distilled water, equilibrated at 6° C., and filtered. The filtrate had its expected toxicity to mosquito larvae greatly reduced or abolished. From this bio-assay it follows that the cuticle or chitin had removed part or all of the DDT. Cuticle or chitin was then added to a more concentrated suspension, equilibrated at 6° C., filtered, and the pieces of cuticle or chitin were soaked twice in cold distilled water and filtered to wash off any DDT that might be present in the water on the surface or within the matrix of the pieces. The lots of cuticle and chitin were then soaked in hot distilled water (90° C.) adjusted to pH 3.8 with 0.1 molar H_2SO_4, filtered, and the filtrate tested by the usual bio-assay method with mosquito larvae. Symptoms and mortality showed that DDT had been recovered. Since DDT could be removed and recovered by these adsorption methods it follows that cuticle and purified chitin are capable of adsorbing DDT.

In addition to the controls accompanying the above adsorption tests, a number of other things were tested. As judged by subsequent bio-assay a small amount of DDT was adsorbed by cleaned dry spines of sea urchins, but none was adsorbed by snail shells, coral, a suspension of erythrocytes (cow), or chunks of muscle (*Limulus*).

Another point which is consistent with the idea that adsorption by chitin facilitates the entry of DDT, is that mosquito eggs are resistant to any concentration of DDT tested, whereas, the larvae on hatching are susceptible. This might represent differences in embryonic stages or a barrier between the egg shell and the enclosed embryo or larva but it could conceivably be due to the fact that larvae have a chitinous cuticle, whereas, the insect egg shell contains no chitin.

If adsorption phenomena are playing an important role, one would expect to find a negative temperature coefficient, i.e., that DDT is more toxic at lower temperatures than at higher temperatures. Solubility tests show that DDT has a positive temperature coefficient for solubility in both polar and apolar solvents. Accordingly, a negative temperature coefficient is good evidence in support of an adsorption hypothesis. Actually the temperature relationships are complex and not fully understood. Data obtained to date show that at low concentrations a negative temperature coefficient is, indeed, obtained but that at higher concentrations this changes to a positive temperature coefficient. However, it seems logical that a concentrating mechanism would be most dominant at lower concentrations. Accordingly, the temperature data support the hypothesis that adsorption of DDT by chitinous cuticles acts as a concentrating mechanism. The shift to a positive temperature coefficient at higher concentrations would seem to imply that other factors are involved and that the adsorption by chitinous cuticles is only for concentrating the toxin, the actual toxic action of DDT being something else.

Most of the temperature studies have been performed using mosquito larvae at 15° and 28° C., but similar results have been obtained with the fresh water shrimp

Gammarus.[7] Lindquist *et al.*, (1945) have independently found a negative temperature coefficient in spray work with adult flies, and during the past year it has come to be generally considered that DDT works better in insect control operations in cool climates than in hot climates. Attempts to obtain data with coelenterates at high and low temperatures failed because we were not successful in keeping the species available at Woods Hole alive in warm, non-running water.

Discussion

From the data presented here, we conclude that the sensitivity of certain animal groups to DDT applied externally as a colloidal suspension in the bathing medium is correlated with the presence of a chitinous or similar cuticle in addition to true differences in tissue susceptibility. This correlation is supported by the similarity of toxicity to certain animals when emulsions are injected in contrast to dissimilarity from external applications, and by direct demonstration of adsorption of DDT by chitin and chitinous cuticles, and non-adsorption by certain other types of exoskeletons and tissues. The idea that adsorption processes are important is further supported by the demonstration of a negative temperature coefficient for toxicity at lower concentrations. The data seem to concern only the differences in sensitivity and not to explain the toxic action itself. The exceptions recorded herein (e.g., snails) show that the action of DDT is more complex but they do not invalidate the cuticle relationship. Accordingly, we propose as a hypothesis that chitinous cuticles act to selectively concentrate DDT from the surrounding medium by adsorption processes.

This hypothesis is in some respects similar to the old idea of penetration due to partition coefficients. Several authors have invoked the idea of partition coefficients to explain insecticide penetration (Wigglesworth, 1941; Richards and Weygandt, 1945). The present hypothesis for concentrating DDT varies in substituting an active adsorbing interface of chitinous cuticle. On theoretical grounds Hurst (1943) has suggested that the insect cuticle may adsorb toxins, as work by Castle (1936) had already indicated. The data in the present paper, however, give the first direct demonstration that a chitinous cuticle can adsorb an insecticide and that the adsorption may play a role in selective toxicity to different groups of animals.

Since one is inclined to think of the arthropodan cuticle as a solid sheet of material, it is natural to ask where sufficient surface is available for the demonstrated adsorption. A possible clue is given by Castle's (1936) work on the "oriented imbedding" of organic substances in chitin. This is a kind of adsorption process that apparently utilizes the surfaces of known intermicellar spaces. Perhaps the same intermicellar surfaces may be available for the adsorption of DDT.

Obviously, adsorption by the cuticle can be only part of the story of the action of DDT. Some mechanism is needed to transport the DDT from the cuticle to its effective locus inside the animal. We have no direct evidence as to what this mechanism is. Perhaps one can interpret the data from distribution of the radio-

[7] More recently Dr. Hsing Yun Fan has made an intensive study of the negative temperature coefficient using both mosquito larvae and midge larvae (*Chaoborus* sp.). His data will be published subsequently.

active bromine analog (Hansen, Hansen, and Craig, 1944) to mean that DDT is more soluble inside insects than in water. Such a difference in solubility in connection with an actively adsorbing cuticle could account for a selective accumulation in insects and other groups with similar cuticles.

A careful consideration of reported exceptions is necessary. Many papers have been published on the use of DDT as an insecticide.[2] These papers, while showing a general susceptibility of insects to DDT, are mostly not comparable to the present paper. They are not primarily concerned with the resistance or susceptibility of the various species but with the practicability of controlling pest insects under natural conditions. A susceptible insect that is not reached by the insecticide in the field will not be killed. Also the thickness, dryness, nature of the epicuticle (external layer outside of chitinous cuticle), and other properties of the cuticle vary greatly from one group to another within the arthropods (Richards, 1944). One should expect variation in susceptibility with these variables. In some cases it seems clear that forms which are not satisfactorily controlled are nevertheless susceptible. For instance ticks and mites are said not to be well controlled by DDT (Cox, 1945) yet they are by no means resistant (e.g., Gouck and Smith, 1944; Smith and Gouck, 1944; Vargas and Iris, 1944, etc.), and at least one species (*Rhipicephalus sanguineus*) is readily controlled by DDT. In some other cases, notably certain beetles with thick, hard, dry cuticles, insects seem little or not at all affected. Since these are terrestrial species, the difficulty in obtaining data comparable to data presented in this paper is considerable. In the Crustacea there is a considerable range of variability; most species tested were highly susceptible but the copepods are much less susceptible (see also Anderson, 1945; Seagren, Smith, and Young, 1945). It seems certain that more and less resistant species do occur among arthropods even though available data refer principally to practical field tests. While it is possible to offer hypothetical explanations of these variations on the basis of differences in cuticle structure, variations in degree of effect seem to be a factor also (Chadwick, 1945). However, the known susceptibility of arthopods in general is so broad that we feel inclined to suggest that the variations will be explained without vitiating the hypothesis advanced in the present paper.

The fungi with chitinous cell walls (*Rhizopus*, etc.) are a true exception. They are apparently indifferent to DDT in the medium bathing them. This resistance cannot be evaluated until we are more certain as to the method by which DDT kills. One obvious possibility lies in the differences between plants and animals, particularly, the absence of a nervous system in plants.

Another line of exceptions concern the intermediate susceptibility of fishes (Eide, Deonier, and Burrell, 1945; Ginsburg, 1945, and others). No explanation of this is offered, but there is no reason to assume that intermediation of a chitinous cuticle is the only means by which DDT can enter an aquatic animal.

The most serious exceptions, however, are those invertebrates which are not affected by the injection of emulsions (snails). These are species representative of a group unaffected by external applications. No explanation of this exceptional lack of effect on injection is offered in the present paper, but obviously the snails demonstrate beyond question that more is involved in the whole story of DDT action than just the facilitation of entrance by an appropriate cuticle.

Summary

1. Throughout the animal phyla there is a correlation between the presence of a chitinous cuticle and susceptibility to external applications of DDT. Those aquatic animals with a chitinous cuticle (arthropods and certain coelenterates) are highly sensitive to external applications of DDT, other animals are not so susceptible although there is considerable variability.

2. The correlation to a chitinous cuticle is supported by studies on various coelenterates, on adsorption, and on temperature coefficients. Coelenterata with respectively a complete, partial, and no chitinous perisarc are respectively highly sensitive, somewhat sensitive, and nearly insensitive to DDT. DDT can be adsorbed by chitin and chitinous cuticles and at low concentrations shows a negative temperature coefficient for toxicity to arthropods.

3. From these data a hypothesis is proposed that chitinous cuticles facilitate the entry of DDT into the animal body by selectively concentrating the compound by adsorption phenomena.

4. This is the first demonstration that an insecticide can be adsorbed by chitinous cuticles and the first direct evidence that such adsorption actually can play a role in insecticide action.

5. The present paper does not consider the nature of the toxicity of DDT to protoplasm. The data given here refer only to the selective action of DDT as a function of penetration facilitated by chitinous exoskeletons. The shift to a positive temperature coefficient at higher concentrations, the lack of effect from injecting DDT emulsions into snails, and the variability in median lethal doses for injected DDT emulsions in different animals, all indicate that the selective adsorption of DDT by chitinous cuticles is only a part of the story of the toxic action of this compound.

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PROTOPLASMIC VISCOSITY CHANGES IN DIFFERENT REGIONS OF THE GRASSHOPPER NEUROBLAST DURING MITOSIS

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Protoplasmic viscosity has been studied by a variety of methods. Each has certain advantages and certain limitations, with regard to the kind of living material to which it is best suited and the accuracy of the results that it will give. These methods and their uses have been reviewed critically by Heilbrunn (1928, 1943) and therefore will not be considered in detail here. Of the several that have been employed the brownian movement method is probably best suited to viscosity studies involving limited regions of the single cell. It may be applied in either of two ways: 1) to calculate the absolute viscosity by measuring the displacement in one direction of the molecularly-bombarded particle and the time required to bring this about, certain other characteristics of the cell and its environment being known, or 2) to compare the viscosities of different regions of a cell at different mitotic stages or under different experimental conditions through observations of the relative speeds of brownian movement. The latter has been used in the present investigation.

The neuroblasts of the grasshopper embryo possess several advantages in such a study. Since they are situated on the ventral side of the embryo, they can be brought next to the cover glass in hanging-drop preparations and microscopic observations of the mitotically active, living cells can extend over several hours. All the cell features that are visible in the usual fixed and stained preparation can be seen, and many phases of the mitotic cycle can be identified readily. The cell is relatively large, measuring about 30 μ in diameter. It maintains a visible polarity from one mitotic division to the next, so that a given region can be located in any cell at any stage of division. The cytosome contains large numbers of mitochrondria, which not infrequently find their way into the spindle during mitosis. By observing the brownian movement of these tiny bodies it is possible to compare the viscosities of the surrounding protoplasm of all non-nuclear parts of the cell at all stages of the mitotic cycle.

Material and Methods

Embryos of *Chortophaga viridifasciata* (De Geer) were prepared by the hanging-drop method previously described for this material (Carlson and Hollaender,

[1] The preliminary observations on which this study is based were made as Rockefeller Fellow in the Natural Sciences at the Genetics Laboratory of the University of Missouri in the winter of 1940–41 and at the Biological Laboratory, Cold Spring Harbor, in the summer of 1941. The work has been completed with the aid of a 1944–45 grant from the University Research Committee of the University of Alabama.

1944). The culture medium, which must be isotonic with the embryonic cells, is made up as follows:

NaCl	0.70	gm.
KCl	0.02	gm.
$CaCl_2$	0.02	gm.
$MgCl_2$	0.01	gm.
NaH_2PO_4	0.02	gm.
$NaHCO_3$	0.005	gm.
Glucose	0.80	gm.
H_2O (pyrex redistilled)	100.0	cc.

The pH of this solution is approximately 6.5, which is about the pH of the grasshopper egg yolk. A small amount of yolk is added to each hanging-drop to provide nitrogenous food materials for the cells.

Preparations were studied in a constant temperature box enclosing all of the microscope except the upper part of the body tube and arm. All observations were made at $26 \pm 0.5°$ C. The light used as a source of microscope illumination was passed through copper sulfate solution to filter out the heat.

Observations were made exclusively on neuroblasts. These cells are shown in representative mitotic stages in Figure 1, which is based on camera lucida sketches of living cells. It will be noted that the neuroblast deviates from the typical cell in two main respects. First, the nucleus is not spherical but roughly hemispherical with a central cytoplasmic core connecting the polar and apolar regions of the cell (Figs. 1*A* and 1*B*). Second, the cell divides unequally to form a small daughter ganglion cell and a large daughter neuroblast (Fig. 1*J*.) The great advantage of these cells is their large size, which makes it possible to observe and study in the living, unstained cell many structural features that are very difficult or even impossible to deal with in smaller cells.

The mitochondria of the neuroblast are spheroidal in shape and measure approximately 0.3–0.5 μ in diameter.

Terminology

Relation of viscosity to brownian movement.

The relation between the rate of brownian movement and viscosity is given by Einstein's equation

$$D_x^2 = \frac{RTt}{N3\pi\eta a}$$

in which D_x is the displacement of the particle along the x axis; R, the gas constant; T, the absolute temperature; t, the time; N, the Avogadro number; a, the radius of the particle; and η the viscosity expressed in poises. Accordingly, the viscosity of the dispersion medium varies inversely as the square of the displacement of a given particle along one axis.

The rapidity of brownian movement of the mitochondria in selected regions of the cell was observed and classified according to the following system. The

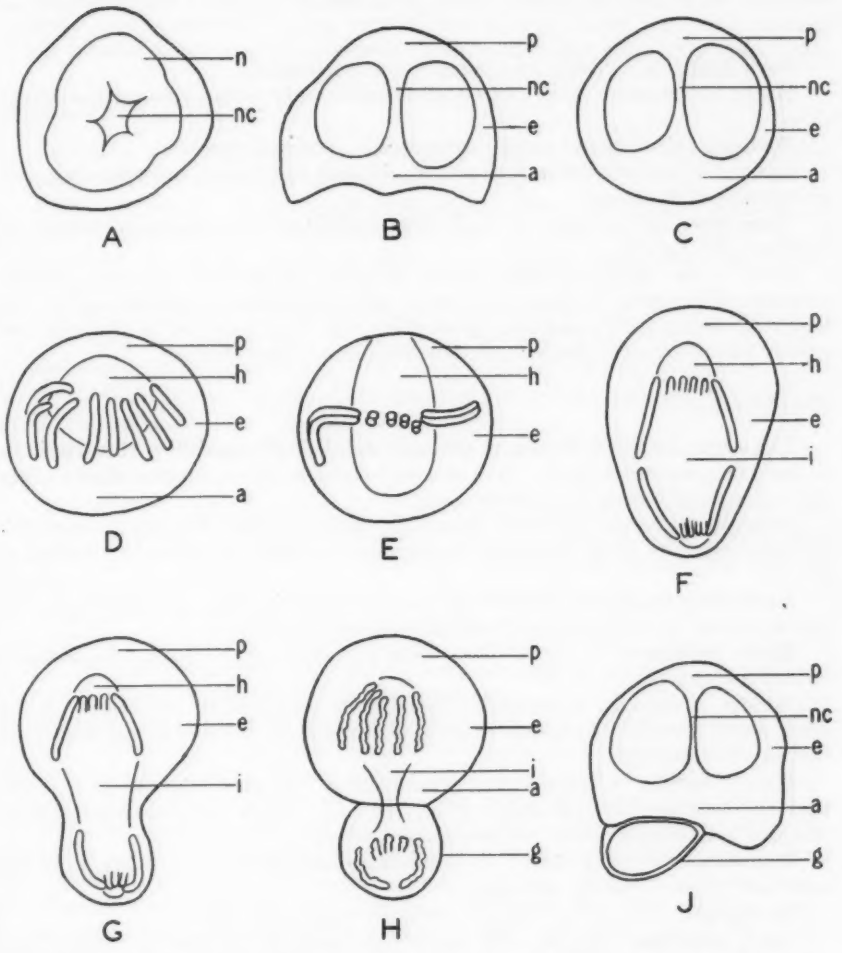

FIGURE 1. Representative stages in the mitotic cycle of the grasshopper neuroblast, reconstructed from camera lucida sketches of living cells growing in culture medium. *A* and *B*, polar and side views, respectively, of interphase or prophase cells; *C*, late prophase a few minutes before breakdown of the nuclear membrane, the cell having acquired a spherical form; *D*, prometaphase; *E*, metaphase; *F*, middle anaphase; *G*, late anaphase; *H*, early telophase; *J*, late telophase, the cell having lost its spherical form. *a*, polar cytoplasm; *e*, equatorial cytoplasm; *g*, ganglion cell; *h*, half spindle; *i*, interzonal region; *n*, nucleus; *nc*, nuclear core cytoplasm; *p*, polar cytoplasm.

viscosity values and the relative speeds of brownian movement are designated as follows:

Very high: No visible movement of mitochondria.

High: Movements very limited, discernible only with prolonged observation.

Medium: Movements readily observable, but not rapid.

Low: Movements quite free and rapid, but individual mitochondria can be followed with the eye.

Very low: Movements so rapid that individual mitochondria cannot be followed with the eye.

Even if the ability to make purely objective distinctions between adjacent members of this series be open to question, values separated by one class, viz., very high and medium, high and low, or medium and very low, can be distinguished readily and would give results essentially similar to those obtained.

Mitotic stages

The stages for which data were obtained and their distinguishing characteristics in the living, untreated state, when observed through 12.5 × compensating oculars and a 2 mm. oil immersion objective, are:

Interphase: Nuclear threads appear in optical section like small, rounded, scattered granules in an otherwise homogeneous nuclear background. Period of growth.

Very early prophase: Nuclear granules smaller in size. Threads have begun to appear in the previously homogeneous background.

Early prophase: Nucleus filled with extremely fine chromatin threads. No nuclear granules.

Middle prophase: Chromatin threads of appreciable thickness. Ends when about seven chromosomes in cross-section can be counted in one-fourth the nuclear circumference.

Late prophase: Chromosomes well-formed. Toward the end of this period cell assumes spherical shape (Fig. 1*B*, 1*C*). Ends with the simultaneous disappearance of the nuclear membrane and appearance of the spindle.

Prometaphase (Fig. 1*D*): Chromosomes straighten and move into the equatorial plane. Spindle develops.

Metaphase (Fig. 1*E*): Chromosomes in equatorial plane.

Early anaphase: Begins with the initial separation of the chromatids and ends when the distal ends of the chromosomes leave the cell equator.

Middle anaphase (Fig. 1*F*): This stage is terminated as the cleavage furrow penetrates to the interzonal region.

Late anaphase (Fig. 1*G*): Ends as the chromosomes lose their sharp outlines and the cleavage furrow appears to be complete.

Early telophase (Fig. 1*H*): This stage lasts until the nucleoli become visible.

Middle telophase: The nucleoli increase in size while retaining their spherical shape, the nuclear membrane re-forms, the interzonal spindle remnant disappears, and the cell re-assumes the hemispherical form.

Late telophase (Fig. 1*J*): Begins as the outlines of the nucleoli become irregular. Ends as linear arrangement of chromatin granules is lost.

The relative duration of these stages is given in an earlier paper (Carlson, 1941).

Regions of cell

Data have been obtained for six different regions of the cell, which are labeled in representative stages of the mitotic cycle in Figure 1.

Polar cytoplasm: This includes the region about the spindle pole of the daughter neuroblast when a spindle is present. At other stages it is the region within which the spindle pole of the daughter neuroblast will develop preparatory to the next division.

Apolar cytoplasm: The cytoplasm situated near the place of formation of the preceding cleavage furrow. It is opposite the polar region.

Equatorial cytoplasm: This is the portion of the cell that is situated at or near the cell equator in metaphase and anaphase and midway between the polar and apolar regions during the remainder of the mitotic cycle.

Nuclear core cytoplasm: The cytoplasmic core that passes through the nucleus (see p. 110).

Half spindle: This includes the developing spindle at prometaphase, the fully formed spindle at metaphase, and at anaphase the portion of the spindle lying between the pole and the plane in which the kinetochores of the adjacent chromosome group are situated.

Interzonal region: This is the part of the cell lying inside the ring of interzonal fibers and situated between the two separating groups of daughter chromosomes. It makes its appearance as the chromatids separate at early anaphase and persists as a spindle remnant into middle telophase.

Nucleus: No information that seemed reliable was secured for this part of the cell. Tiny nuclear granules comparable in size to the mitochondria showed very limited movement at various stages of the mitotic cycle. This might indicate a persistently high viscosity or it might mean merely that these granules are attached to the chromatin threads, which form a semi-rigid framework within the nucleus, and would therefore not be expected to exhibit much brownian movement. Bělař (1929a), however, has reported a rapid brownian movement of tiny granules of unknown nature situated in the ground substance of the nucleus of the grasshopper spermatocyte during prophase and telophase, indicating, according to him, that this substance is quite fluid.

Observations

The curves shown in Figure 2 were constructed in the following way. A linear series of arbitrary numerical values was assigned to the five selected viscosity classes described previously. Four observations were made for each region of the cell on different days and with different preparations. These were averaged and plotted. Because the determination of each of these was to a certain extent subjective, in that it depended on the judgment of the observer, and because the vis-

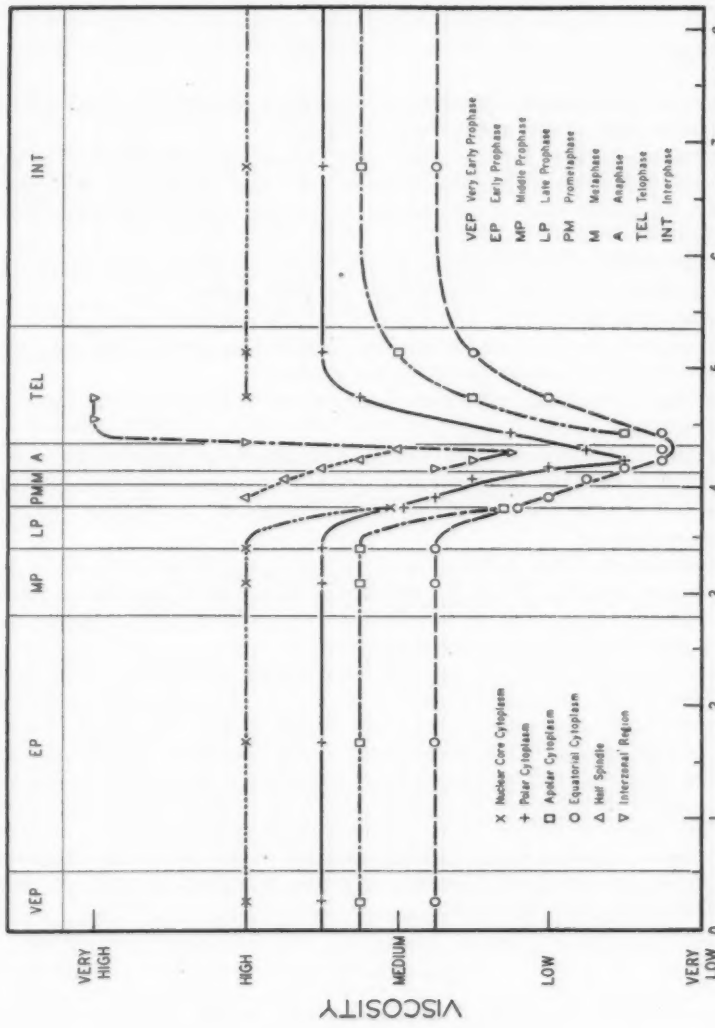
VISCOSITY
VERY HIGH
HIGH
MEDIUM
LOW
VERY LOW
TIME IN HOURS
VEP
EP
MP
LP
PMM
A
TEL
INT
0
1
2
3
4
5
6
7
8
X Nuclear Core Cytoplasm
+ Polar Cytoplasm
□ Apolar Cytoplasm
○ Equatorial Cytoplasm
△ Half Spindle
▽ Interzonal Region
VEP Very Early Prophase
EP Early Prophase
MP Middle Prophase
LP Late Prophase
PM Prometaphase
M Metaphase
A Anaphase
TEL Telophase
INT Interphase

FIGURE 2

cosity varied slightly from preparation to preparation and from cell to cell, certain of these curves showed irregularities. In order to discover whether these were the result of experimental error or of actual viscosity shifts, new cultures were prepared, checked, and any necessary corrections made. It is believed that the graph in its present form represents quite accurately the cycle of viscosity changes in the different regions of the cell during mitosis. It should be emphasized, however, that the five arbitrarily-chosen viscosity values represented by ordinates are not necessarily a linear series of absolute values, as their graphical treatment implies.

During the greater portions of interphase and prophase the viscosity of the cytosome maintains a relatively high and constant value. In late prophase, a few minutes before the disappearance of the nuclear membrane, a rapid fall in viscosity is initiated. This change begins as the cell changes in shape from hemispherical (Fig. 1*B*) to spherical (Fig. 1*C*). The increase in the rapidity of mitochondrial movement is distinguishable first at the juncture of the equatorial and apolar regions as this part of the cell begins to draw in coincidentally with the start of the rounding-up process. From this region the lowered viscosity spreads into the apolar region, which thickens as the rounding-up proceeds, and subsequently into the equatorial and polar regions. Obviously, some of this is due to the actual movement of the cytoplasm, which must of necessity shift in position as the cell shape changes, but this can hardly be responsible for more than a small fraction of the viscosity change that takes place at this time. Since this alteration in shape occupies only about 5–10 minutes, this sequence of changes does not appear in the graph. The viscosity continues to fall through prometaphase and metaphase to reach a minimum in anaphase. The progress of the cell through telophase is accompanied by a steady rise in viscosity until the interphase-prophase level is attained. During telophase the hemispherical form of the cell is re-assumed (Fig. 1*J*).

The half spindle appears to show a slight fall in viscosity from prometaphase to early telophase, by which time it is too small for further study.

As the chromosome halves begin to separate at early anaphase, the interzonal region between the groups often contains one or more mitochondria showing a moderate amount of brownian movement. During middle and late anaphase large numbers of mitochondria move into the interzonal region, until they are as concentrated as outside, except for a small region inside each of the separating chromosome groups. Their movement is quite rapid, but less so than that of the mitochondria outside in the equatorial region. As the cleavage furrow presses in against the interzonal fibers, the transverse diameter of the interzonal region decreases and the motion of the mitochondria quickly slows down and then stops entirely. The fact that these stationary bodies are lined up in rows parallel with the long axis of the spindle makes it appear as if they had been caught among interzonal fibers of higher viscosity as these were pressed inward by the deepening cleavage furrow. By late telophase these fibers are no longer visible.

The high viscosity level of the nuclear core cytoplasm immediately after the formation of the nucleus and core may be due to the fact that this is the region occupied earlier by the spindle remnant of the preceding division.

Discussion

The brownian movement method

Of the three most frequently used methods of making determinations of protoplasmic viscosity changes, viz., centrifuge, microdissection, and brownian movement, the last has the advantage of producing no physical disorientation of the cell contents. It can be depended on to give reliable results, however, only if certain factors are taken into consideration:

1) The protoplasm surrounding the granules in brownian movement must be homogeneous, if the viscosity of the protoplasm as a whole, exclusive of the granules, is being determined. If regions of different viscosity are present, observations of the rate of brownian movement of a particle will indicate only the viscosity of the material immediately surrounding it. The polar, equatorial, apolar, and nuclear core regions of the neuroblast cytosome are visibly homogeneous except for the mitochondria, and the fact that these bodies appear to migrate about and pass one another in the course of their shorter zig-zag movements demonstrates that they are not enclosed in a substance of one viscosity that is in turn surrounded by material of another viscosity. The spindle, however, is probably an exception to this (see p. 118).

2) Alterations in the size of the mitochondria from one stage of mitosis to another would cause an apparent shift in the viscosity of the surrounding medium, even though no actual change took place. Such changes could conceivably result either from shrinking and swelling as a consequence of osmotic shifts or from division and growth. The latter can be ruled out in the case of the neuroblast because very few mitochondria would need to divide in each cell generation to make up for the loss to the daughter ganglion cell, which receives very little cytoplasm and very few mitochondria. The resulting effect on brownian movement would be insufficient to alter the general viscosity picture. With regard to osmotic changes, Lewis and Lewis (1915) report that immersing cells in hypotonic solution causes swelling of the mitochondria. Bělař (1929a), on the other hand, states that mitochondria are particularly insensitive to the usual swelling effects of hypotonic solution. Unfortunately, the neuroblast mitochondria are so small and move so rapidly at certain stages that exact comparisons of size by actual measurements are impossible. Careful visual comparisons of the mitochondria of adjoining cells in different mitotic stages, however, have failed to reveal any significant size differences. As far as the present study is concerned, swelling of the mitochondria of late prophase, prometaphase, and metaphase cells by intake of water (p. 119) would tend to reduce rather than augment the observed viscosity shift in these stages; therefore, the actual change would be even greater than that shown in Figure 2.

3) If the granules under observation are so crowded as to interfere with each other's movements, the viscosity values obtained at the lower levels will be too high; for frequent collisions will retard their movements. The mitochondria of the neuroblast are definitely crowded, and this could easily result in appreciable errors in absolute viscosity values based on the migration of a given body a certain distance in a certain time, according to the method described by Pekarak (1930). Much of this error is doubtless avoided in the present study, however, because viscosity determinations were based on the rapidity with which the particles as a group dance about rather than on the total distance they migrate in a given interval of time.

Results of related studies

Of the several studies made on viscosity changes of the whole cytoplasm during mitosis, the centrifuge determinations of Heilbrunn (1917, 1921) for the marine invertebrate egg (Arbacia, Cumingia, and Nereis) and of Kostoff (1930) for meiotic cells of Nicotiana and the brownian movement determinations of Kato (1933) for the meiotic cells and staminate hairs of Tradescantia have given results that are similar to those I have obtained for the polar, apolar, and equatorial cytoplasm of the grasshopper neuroblast. All these studies show that the viscosity is relatively high during interphase, when interphase is referred to at all, high during all or most of prophase, falling during metaphase, lowest at anaphase, and rising at telophase.[2] The similarities in these results are all the more striking in view of the great diversity of the cell types used. The marine invertebrate egg contains relatively large quantities of ergastic matter, such as oil globules and yolk and pigment granules, and develops during mitosis an astral system and spindle that involve a large portion of the protoplast. The pollen mother cell, though consisting of a more or less homogeneous cytosome with relatively small spindle and no astral system, nevertheless is atypical mitotically because of the extended prophase and the two successive meiotic divisions without an intervening interphase. The staminate hairs of the plant contain large vacuoles with only a peripheral film and a few central strands of cytoplasm. The grasshopper neuroblast contains a fairly homogeneous cytosome with no visible ergastic matter or vacuoles but an abundance of mitochondria. The spindle is moderate in size and no asters are visible. This suggests strongly that viscosity change is a fundamental factor in mitosis, and is largely independent of individual pecularities of the type of cell studied.

Zimmermann (1923), who studied the viscosity of the alga, Sphacelaria, by means of the centrifuge and brownian movement, and Seifriz (1920), whose studies of the marine invertebrate egg are based on microdissection, place the viscosity fall and minimum somewhat in advance of this mitotically. According to Zimmermann the viscosity is lowest at metaphase, while Seifriz finds it lowest during late prophase and metaphase. Both describe the viscosity as rising at anaphase and high at telophase.

In contrast with these findings are the results obtained by Chambers (1917, 1919) from studies of Arbacia and Echinarachnius eggs with the microdissection needle. He reported that the viscosity of the greater part of the cell is low during prophase, rising during metaphase, high during anaphase and early telophase, and falling in late telophase. He related the viscosity to the state of development of the amphiaster: low when no amphiaster is present, rising as the amphiaster forms, highest when it has reached its maximum development, and falling as the amphiaster disappears at the end of mitosis. He does, however, describe a liquefaction of the equatorial region previous to anaphase and persisting through cleavage.

Fry and Parkes (1934) duplicated as closely as possible the centrifuge studies of Heilbrunn on Arbacia, Cumingia, and Nereis eggs. The results they obtained for viscosity in relation to time after fertilization were identical with those reported earlier by Heilbrunn; in fact, in their paper they used Heilbrunn's curves for Arbacia and Nereis eggs. They interpreted this data, however, as supporting

[2] Heilbrunn associates the pre-cleavage fall and the post-cleavage rise in viscosity of the Arbacia egg with spindle development and disappearance, respectively.

Chambers' conclusions, claiming that Heilbrunn had misidentified certain of the mitotic stages. This would place viscosity changes of the marine invertebrate egg in entire disagreement with those of the grasshopper neuroblast, in which there can be no question of the correct identification of the different mitotic stages.

Kostoff's description of viscosity changes in somatic cells of Nicotiana (1930) is not in accord with any of these results. Using the centrifuge technique, he reported two cycles of viscosity change for each mitotic cycle: high viscosity at prophase and anaphase, and low viscosity at metaphase and interphase.

Observations on the viscosity of the spindle are complicated by the probable presence in the half spindle of two materials, spindle fibers and interfibrillar substance. If there are two such materials present, the mitochondria or other cytoplasmic granules that make their way into the spindle by brownian movement would be expected to occupy the region of lower viscosity, i.e., the interfibrillar region. Under such conditions their speed of movement would be determined by two factors: the viscosity of the interfibrillar substance and the amount of space available for movement between the fibers, especially if this were very limited.

Bělař (1929b) found that most spindles in cells of the stamen hairs of Tradescantia contained a few tiny granules in quite rapid brownian movement. This motion of the granules, which appeared first in the polar caps (clear regions that adjoin opposite sides of the nucleus at late prophase and in which the spindle later develops), was evident in the fully-formed spindle and continued undiminished up to the time of formation of the cell plate, when the granules disappeared from view. This contrasted with the situation he found in the spindles of animal cells, namely, nematode eggs, lepidopteran and grasshopper spermatocytes, and Actinophrys, in which granules of a comparable size in the spindle were always relatively quiet (see Bělař, 1929a). He interpreted this difference to indicate that in the Tradescantia cell there was a larger amount of the less viscous interfibrillar substance than in the animal cells he studied. I have not been able to confirm Bělař's observation that the granules show greater freedom of movement in the direction of the long axis of the spindle than at right angles to it, but this may mean only that the interfibrillar substance is more abundant and the fibers farther apart in my material than in his.

Ris (1943) reports unrestricted brownian movement of the cytoplasmic granules that make their way into the interzonal region during anaphase in embryonic cells of Tamalia. Chambers (1924) states that the spindle of the dividing sand-dollar egg has become "distinctly fluid" by the time the chromosomes have reached the poles. He doubtless refers to what I have termed the interzonal region. These observations agree well with the conditions in the grasshopper neuroblast.

Possible causal factors in viscosity change

With regard to the factors responsible for viscosity changes of the cytoplasm, two possibilities seem deserving of consideration: alterations in the water content and in the pentose nucleic (ribonucleic) acid content of the cytoplasm.

It has already been pointed out (p. 115) that the first detectable viscosity drop coincides with the initiation of the "rounding-up" of the neuroblast in late prophase and that the viscosity is rising as the cell flattens on one side to assume the hemispherical form during telophase. Since it can be demonstrated that immersion of

these cells in hypotonic culture medium causes a fall in viscosity owing to intake of water and that hypertonic medium causes a rise in viscosity through loss of water, it seems not unlikely that the late prophase viscosity fall and "rounding-up" of the cell might result from the intake of water, while the telophase viscosity rise and the accompanying flattening of the cell at one side might be due to loss of water. Unfortunately, the irregularities in the hemispherical-shaped cell make it impossible to determine whether there is any actual change in cell volume during the alteration in shape. Chambers (1919) relates the change in form of the Arbacia egg from spherical to hemispherical following the first cleavage division to a viscosity shift, but he associates it with a lowering of the viscosity.

Water exchange seems inadequate, however, as a complete explanation of the observed viscosity changes for two reasons. First, it seems doubtful that the intake of water would occur in sufficient amounts to account entirely for the observed viscosity fall. Second, the viscosity continues to rise for some time after the cell has returned to its hemispherical shape, when the water loss might be supposed to have been completed. Another factor, therefore, would appear to be involved, either in place of or in addition to water intake and loss.

In support of the possibility that changes in the pentosenucleic acid content of the cytoplasm may be at least partly responsible for the observed viscosity changes is the fact that pentosenucleic acid, which is known to be present in large quantities in the cytoplasm of rapidly growing tissues (Brachet, 1933; Caspersson and Schultz, 1940) and which has a strikingly high viscosity ($\eta = 62.4$, according to Cohen and Stanley, 1942), undergoes a change in amount per cell during mitosis (Brachet, 1940; Caspersson, 1940; Painter, 1943). Both Brachet, from studies of the eggs and early developmental stages of different animals, and Painter, using Rheo meiotic cells, reached the conclusion that cytoplasmic pentosenucleic acid is abundant in mitotically active cells at early prophase, less abundant or entirely absent from late prophase through anaphase, and increasing in amount following division. They believe that the late prophase decrease is due to transformation of pentosenucleic acid into the desoxypentosenucleic acid of the developing chromosomes and that the increase following division is due to the loss of desoxypentosenucleic acid from the chromosomes and its transformation into pentosenucleic acid. These changes in the nucleic acid content of the cytoplasm during mitosis bear a very close resemblance to my viscosity curve for the neuroblast cytoplasm. If there is any discrepancy, it would seem to be the exact time during late prophase that the nucleic acid leaves the cytoplasm in detectable amounts, and neither the studies of Brachet nor Painter furnish information on this point. The statement of White (1942), however, that "the nucleic acid of the chromosomes undergoes a sudden increase at prometaphase, when the nuclear membrane breaks down and substances from the cytoplasm have free access to the chromosomes" suggests that the fall in cytoplasmic nucleic acids may coincide closely with the viscosity fall of the cytoplasm. The viscosity rise, which extends through all of telophase, could be accounted for, up to the time of nuclear membrane formation, by a return of the pentosenucleic acids to the cytoplasm during the retrogressive chromosome changes, and, after re-constitution of the nuclear membrane, by the synthesis of new pentosenucleic acid.

SUMMARY

Observations on the rapidity of brownian movement of the mitochondria in different regions of the grasshopper neuroblast during the entire mitotic cycle indicate that:

1) The viscosity of all parts of the cytosome is relatively high during interphase and prophase, begins to fall in late prophase, reaches a minimum at anaphase, and rises gradually to its original high level during telophase.

2) The viscosity of the portion of the spindle between the pole and the plane in which the proximal ends of the chromosomes are situated appears to fall slightly from a high prometaphase level through anaphase.

3) The viscosity of the portion of the spindle situated between the separating daughter chromosome groups shows, during anaphase, a slight drop from a medium value, and this is followed at the beginning of telophase by an abrupt rise to a very high level, which is maintained through early and middle telophase.

Alterations in water content and nucleic acid content of the cytoplasm are suggested as possible explanations of viscosity changes during mitosis.

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TOXIC EFFECTS OF COPPER ON ATTACHMENT AND GROWTH OF BUGULA NERITINA [1]

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Introduction

This paper describes physiological experiments dealing with the effect of copper on attachment and early development of *Bugula neritina* (L.), a widely distributed marine fouling organism. The studies were designed to gain a clearer understanding of the specific role of copper in prevention of fouling and the mechanism of action of copper paint surfaces. Various experiments using copper solutions and paints were made to gain an integrated concept of copper toxicity.

Copper, in one form or another, has been used since ancient times to prevent fouling and is one of the most effective metallic poisons for marine fouling organisms (Visscher, 1927). The antifouling efficiency of metallic poisons is related to the intrinsic toxicity of their ions and the solubility of their corrosion products in sea water, both relatively high for copper (Parker, 1924). Recently, this concept has been clarified and extended by Ketchum, Ferry, Redfield, and Burns (1945) who show that the prevention of fouling is related to the concentration of toxic dissolved in the water at the surface of the paint, and that the rate of loss of toxic is a measure of the steady-state concentration in a narrow zone at the paint surface. The rate of loss of toxic from a paint surface is known as the leaching rate. The authors show that in the case of copper paints, a leaching rate maintained consistently above a minimum of 10 micrograms of copper per square centimeter per day will prevent serious fouling.

Much discussion and some experimentation has centered around the question of the relative toxicities of different forms of copper. Carritt and Riley (1943), however, were unable to demonstrate any difference in toxicity to *Bugula turrita* between cuprous and cupric ions, while Clarke (1943) found that "all forms of copper have roughly the same toxicity but differ greatly in their solubilities."

The establishment of fouling organisms on a submerged surface depends *a priori* on their ability to affix themselves and to develop after attachment. Effective antifouling surfaces, therefore, must function in one or both of two ways, by repelling (or killing) larvae of fouling organisms, or by inhibiting development of any that attach. Visscher (1927), Neu (1932), and Edmondson and Ingram (1939)

[1] These studies were conducted at the Naval Biological Laboratory at San Diego, California, under direction of Mr. W. F. Whedon to whom acknowledgment is due for many suggestions. The author is indebted to Dr. Alfred C. Redfield of the Woods Hole Oceanographic Institution for helpful advice and criticism. Acknowledgment is also made to Mr. C. J. Rapean and Miss Mildred Marshall for making the chemical analyses, and to Dr. Easter C. Cupp for valuable assistance. The Navy Department has given permission to publish the results. The opinions contained herein do not necessarily reflect the official opinion of the Navy Department or the naval service at large.

conclude from indirect evidence obtained mainly in field studies that toxic paints have no effect on growth of fouling organisms once attached, that they function only by repelling or delaying attachment. Without minimizing the repellent action of antifouling paints, evidence is accumulating to show that toxic surfaces function importantly through inhibition of growth of attached larvae. The ingenious experiments of Pomerat and Weiss (1943) showing inhibition of fouling growth on unpainted areas by adjacent toxic paint surfaces strongly support this view. Similar "distance effects" of antifouling paint surfaces on fouling growth on adjacent untreated areas are frequently seen in panel tests (e.g., Edmondson, 1944, Fig. 7a, p. 13).

Toxic effects of copper on various fouling organisms have been described by Bray (1924), Whedon *et al.* (1942 and 1943), Miller and Cupp (1942), Clarke (1943), and Riley (1943) in unpublished reports to the Bureau of Ships, U. S. Navy Department. Prytherch (1934) reports stimulating effects of small amounts of copper on settling and metamorphosis of the oyster, and similar oligodynamic effects on ascidians were observed by Grave and Nicholl (1939).

Jones (1941) reviews theories on the mechanism of toxic action of metals, notably copper. These may be divided into two main groups which maintain that: (1) copper retards vital processes through inactivation of essential enzymes, and (2) copper acts more directly by precipitating cytoplasmic proteins as copper-proteinates. With regard to fouling organisms, Clarke's (1943) studies on barnacles and mussels, and Riley's (1943) investigations and those of the present author on bryozoans favor the inactivation theory. Further studies are needed, however, to elucidate the physiological mechanisms involved.

The results of the present investigation will be presented in four parts: (1) attachment and growth of *Bugula* on copper paint surfaces, (2) growth of *Bugula* in copper solutions, (3) recovery of *Bugula* from copper poisoning, and (4) toxicity gradients of copper paint surfaces.

Materials and Methods

Bugula larvae for these studies were obtained as follows. Mature colonies were collected from docks, piling, etc., in San Diego Bay the day before tests were made, and placed in aquaria of sea water in the laboratory. Early the next morning, these liberated swarms of larvae which were easily collected at the light side of the container since they are positively phototropic and visible to the naked eye (about 0.2 mm. in length and darkly colored). *Bugula* larvae normally attach and begin to grow before mid-afternoon of the day on which they are liberated as described by Grave (1930) and Edmondson (1944). In subtropical localities, as San Diego Bay, larvae may be obtained the year around. From the foregoing facts, it is obvious that this form is remarkably suited for studies on fouling problems.

Primed steel, 1 × 3 inch panels were coated with the various experimental and control paints employed in the first section of this paper. Larger panels (10 × 12 inches) were similarly prepared and exposed in the bay to test their antifouling efficiency under field conditions. In most cases, the small panels were "seasoned" before testing by soaking them for a month or more in large jars of sea water. The seasoning bath was changed at frequent intervals and aerated by means of a stream of air bubbles delivered at the base of the container by means of a tapered glass tube.

For other studies, larvae were allowed to attach to small test panels which were coated with a non-toxic paint composed of equal parts of paraffin and ester gum. So attached, they could conveniently be transferred to the experimental situations or be removed therefrom for observations.

Other procedures will be described as necessary in the various sections of the investigation.

Attachment and growth of Bugula on copper paint surfaces

The first problem was to determine the effect of actual copper paint surfaces on attachment and growth of *Bugula* larvae under simulated natural conditions. The data given are typical of many such tests that have been performed.

TABLE I

Attachment and growth of Bugula larvae on hot and cold plastic paints, each series with graded copper content. (Paints seasoned for two months in sea water bath before tests were made.)

Series	Paint No.	Percentage Cu_2O in dry paint	Leaching rate (2-mo.) μg Cu/cm.²/day		Per cent larval attachment ± S.E.		Growth (3-day) Maximum length [1] (mm.)	
			Filmed	Clean	Filmed	Clean	Filmed	Clean
Hot plastic	1	37.0	28.1	24.1	4±1.6	2±1.1	0.3	—
	2	32.0	19.4	23.7	23±5.3	15±4.4	0.3	0.3
	3	26.0	14.0	14.0	5±3.3	13±6.3	0.2	0.2
	4	19.0	13.5	11.5	79±3.5	72±5.0	0.2	0.2
	5	10.6	6.9	5.8	86±4.4	95±2.6	0.4	0.6
	6	0	—	—	100	100	1.3	1.2
Cold plastic	7	36.0	14.3	14.1	0	3±2.4	—	0.2
	8	30.0	14.1	13.3	5±3.0	1±1.4	0.2	0.2
	9	19.2	14.1	13.0	7±2.4	10±4.5	0.3	0.2
	10	9.3	3.5	3.8	87±3.6	73±6.8	0.6	0.7
	11	0	—	—	78±7.4	98±1.4	1.1	1.1

[1] As attached larvae are approximately 0.2 mm. in length, maximum growth increments may be calculated by subtracting this value from the maximum lengths given above.

Two series of copper paints (one hot plastic and one cold plastic series)[2] were prepared, each having the same matrix composition but graded amounts of cuprous oxide (Table I). Two test panels were coated with each of these paints and seasoned for ten weeks. Prior to testing, one panel in each duplicate set was wiped to remove the slime film that accumulated during seasoning; the other was used without being cleaned.

At the start of the attachment test, the panels were immersed in jars containing 700 cc. fresh sea water and immediately thereafter a known number of *Bugula* larvae (usually 100) were added. Tests were always started in mid-morning when the larvae were active, and counts of the number attached were made late in the after-

[2] A hot plastic paint is one that is applied hot and forms a solid film on cooling; cold plastic paints are one of the types of paints which dry by evaporation of the solvent. The paints used in these experiments were experimental modifications of standard Navy formulations.

noon when all larvae were either attached or dead. The percentage of attachment was calculated by dividing the number of larvae affixed to the test panel by the total number of larvae used.

Data on development, which involves both growth and differentiation, were obtained by measuring the length from base to apex of the young *Bugula* stalks, and by determining the number of polypides developed from time to time. Length measurements were made with an ocular micrometer under low magnification.

The results of the attachment and growth tests on the various copper paint surfaces are given in Table I and in Figures 1 and 2. The data clearly segregate two groups of paints. Those with less than 15 per cent Cu_2O content or leaching rates less than 10 micrograms of copper per square centimeter per day are obviously inferior as over 70 per cent of the larvae were able to attach to them *and* to grow significantly. These paints foul in a more or less short period of time in field tests. The second group, with greater copper content and concomitantly higher leaching rates, were significantly more efficient in repelling attachment and permitted no significant growth. These paints are more effective in the field, the length of their effective periods being related to their copper content.

That leaching rate rather than copper content is responsible for the antifouling performance of a paint can easily be demonstrated by comparing *Bugula* attachment and growth on surfaces with identical amounts of cuprous oxide but different leaching rates. If, for example, ester gum is substituted on an equal weight basis for rosin as the resinous component in our hot plastic series, leaching rates fall far below the adequate level and *Bugula* larvae attach abundantly and develop colonies on such surfaces. Ketchum *et al.* (1945) have shown that substitution of ester gum, esterified Albertol or coumarone-indene for the rosin content of an effective antifouling paint (Navy Department Specification 52-P-161) drastically reduces the leaching rate below the critical level with the result that such paints foul quickly in the field. Thus, copper content is important only insofar as it contributes to an adequate and sustained leaching rate, and may be neglected in the subsequent discussion.

In these tests, the slime film on the various paint surfaces had no consistent effect on larval attachment. Its presence apparently is not necessary for attachment of *Bugula* since larvae affixed themselves to cleaned panels as well as those with a coating of slime film, and since they readily attach themselves to unseasoned surfaces shortly after their initial submergence and certainly before any visible film forms. Whether or not an invisible film is prerequisite to attachment is an academic question. In the tests cited, the larvae had no choice between surfaces with and those without slime film. When such choice is involved, *Bugula* larvae do exhibit certain preferences (Whedon *et al.*, 1943). The role of the slime film on attachment to toxic and non-toxic surfaces under various experimental conditions will be considered in another paper.

Larval attachment to paints with leaching rates above 15 micrograms of copper per sq. cm. per day ranged between zero and 23 per cent with an average of less than 10 per cent. None of these was able to grow, however, and hence to establish a colony. The range of leaching rates between 10 and 15 μg. per sq. cm. per day may be regarded as a "marginal zone," especially as the lower value is approached, since paints with leaching rates in this range often permit considerable attachment

of larvae. These may or may not be killed. If not, as will be shown later, some of them might recover and grow should the leaching rate and subsequent surface concentration of toxic fall below the adequate growth inhibiting level. Even if the attached larvae are killed, however, there is still the possibility that their dead bodies might form a less toxic substrate for later larvae than the paint surface

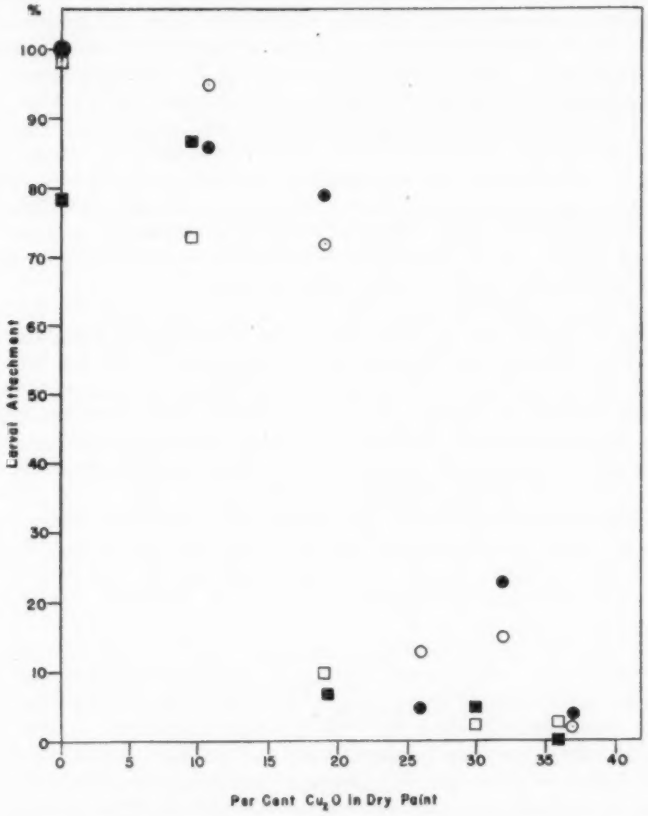

FIGURE 1. Attachment of *Bugula* larvae in relation to copper content of paint. Circles represent hot plastic paints while squares indicate cold plastic paints. Black-filled data points represent paint surfaces coated with slime film while clear data points represent cleaned surfaces (wiped before testing). All paints were seasoned for ten weeks in sea water before testing.

itself. Larvae frequently attach themselves on top of other larvae, often in clusters. Conceivably, those underneath might take up the toxic ions diffusing from the paint or in other ways shield the overlying larvae so that the latter could develop. Certainly, those on top would be further removed from the paint surface and hence would be located in a less concentrated part of the toxic zone which might permit

their development. While this sort of attachment is obviously not very secure, firmer attachment may eventuate by means of stolonic outgrowths from developing colonies to the paint surface. Such root-like processes are developed by erect bryozoans and may give rise to secondary colonies.

The fact that at least a small percentage of larvae attach at any one time to quite toxic surfaces indicates that under field conditions the growth inhibiting func-

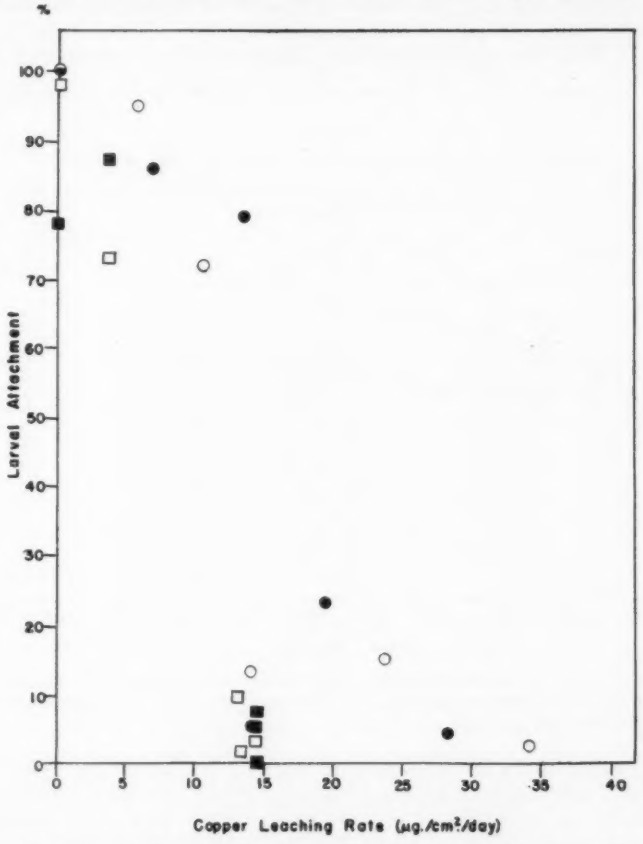

FIGURE 2. Attachment of *Bugula* larvae in relation to copper leaching rate. Symbols as in Figure 1.

tion of toxic surfaces plays an important role in the prevention of fouling. Effective antifouling surfaces must function in part by stopping development of attached larvae. This is contrary to the previously mentioned views of Visscher, Neu, and Edmondson and Ingram. The latter authors report a restricted growth of *Bugula* and serpulid worms on toxic surfaces exposed in Hawaiian waters, but attribute this to "delayed attachment rather than to a slower rate of growth after settling."

Dates of attachment in their tests are not known, and hence rates of growth on treated and untreated surfaces cannot be calculated and compared from their data. In view of the present studies, it would seem that the alternate interpretation of a growth retarding effect is equally plausible, if not more probable.

To illustrate further the growth retarding effect of toxic surfaces, another experiment involving growth of *Bugula* on unseasoned copper paint surfaces may be cited. In this experiment, the first two weeks of growth of *Bugula* on two hot-plastic, copper-paint surfaces (*A* and *B*) was compared to that on a non-toxic, hot-plastic control (*C*). The two toxic paints have identical copper content (32 per cent

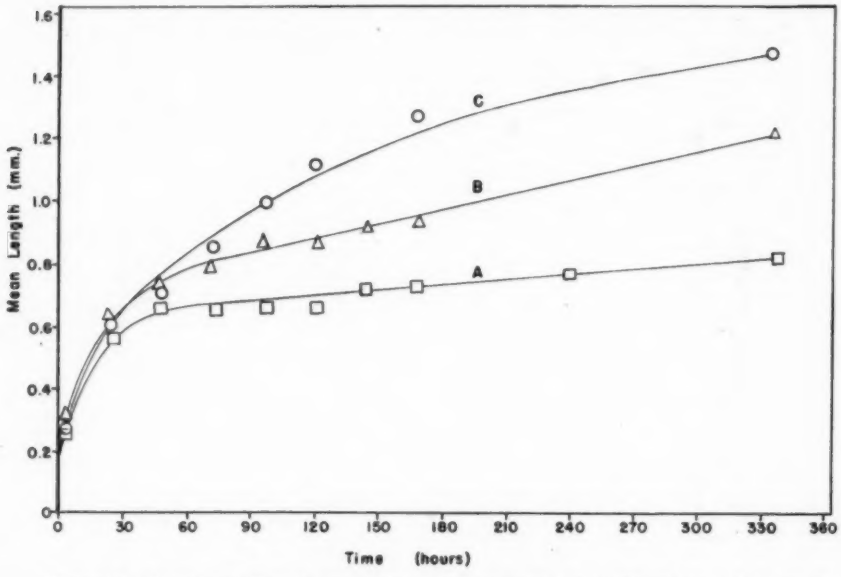

FIGURE 3. Growth of *Bugula* ancestrulae on unseasoned hot-plastic paints. *A* is an excellent copper paint with adequate leaching rate, *B* is an inferior paint with low copper leaching rate, and *C* is a non-toxic control.

Cu_2O), but paint *A* (w.w. rosin-paraffin matrix) has an excellent field record correlated with adequate leaching rates, while paint *B* (ester gum-paraffin matrix) fouls quickly in the field because of low leaching rate. Hot-plastic paints of the type here used have low initial leaching rates and do not develop characteristic leaching rates until they have been submerged for a period of time. Hence, *Bugula* larvae attach abundantly to unseasoned panels coated with these paints and begin to grow normally on them.

As shown in Figure 3, the growth curves in the present experiment do not begin to diverge significantly until after the second day. Growth on paint surface *A* was practically stopped at this time at the first polypide stage. Some individuals developed the first polypide, most did not, and no colonies were established. Colonies were developed on paint surface *B*, but growth and differentiation were sig-

nificantly less than for controls. The three curves become more and more divergent with time.

The foregoing differences between growth inhibiting properties of paints *A* and *B* must be attributed to differences in their leaching rates as their copper content and growth of *Bugula* on their respective controls are identical. Repeated leaching rate determinations of these paints have shown both to have low initial leaching rates (which accounts for the initial similarities in the *Bugula* growth curves for the two surfaces) but paint *A* eventually develops an adequate leaching rate, while leaching rates of paint *B* do not attain the adequate level. Thus, again the growth retarding property of copper paint surfaces is demonstrated and associated with the copper leaching rate.

TABLE II

Effect of copper on growth and development of Bugula ancestrulae

Av. copper concentration mg./liter	Growth and differentiation in copper solutions				
	Mean length increments ± S.E.			Polypides per colony	
	1 day	3 days	5 days	3 days	5 days
	mm.	mm.	mm.		
Controls	0.36±.007	0.61±.016	0.77±.021	1–2	1–2
0–.05	0.30±.015	0.55±.013	0.63±.018	1	1–2
0.05–.10	0.26±.009	0.37±.010	0.46±.012	1	1–2
0.10–.15	0.25±.007	0.36±.008	0.48±.013	1	1
0.15–.20	0.14±.008	0.22±.009	0.24±.011	0–1	0–1
0.20–.25	0.08±.007	0.10±.008	0.12±.010	0	0
0.25–.30	0.02±.007	0.03±.008	0.02±.009	0	0
0.30–.35	0.04±.011	0.03±.011	0.03±.009	0	0
0.35–.40	−0.03±.008	0.01±.009	0±.008	0	0
0.40–.45	0.03±.007	0.02±.011	−0.03±.010	0	0
0.45–.50	0.02±.008	0.03±.010	0.03±.010	0	0

The experiments cited in this section are in close accord with results obtained by Ketchum *et al.* (1945) and substantiate the leaching rate theory of action of antifouling paints. It is noteworthy that their estimate of minimum adequate copper leaching rate (10 micrograms per sq. cm. per day), which was based on comparisons of numerous leaching rate determinations with field exposure tests, is confirmed by the *Bugula* attachment and growth tests. Data presented in this section show that copper paints with leaching rates less than this value permit larvae to attach in large numbers and to grow and differentiate. Paints with leaching rates greater than 10 micrograms per sq.cm. per day allow only a small percentage of larvae to attach and completely inhibit their growth. Large percentages of larvae occasionally attach to paint surfaces with leaching rates between 10 and 15 micrograms of copper per sq. cm. per day, but these do not develop into colonies.

Growth of Bugula in copper solutions

To determine more precisely the effect of copper ions on growth of *Bugula*, nontoxic panels bearing attached larvae (about 25) were immersed in sea-water solutions of graded copper concentrations. Cuprous oxide was the salt used in making

the copper solutions, but the ions were presumably cupric since the oxidation of cuprous ion is supposedly rapid. The young *Bugulae* were measured just before immersion in the experimental solutions and at one, three, and five day intervals thereafter. Polypide development was also observed at these times. Mean length increments after immersion were computed as an index of toxicity of the solutions.

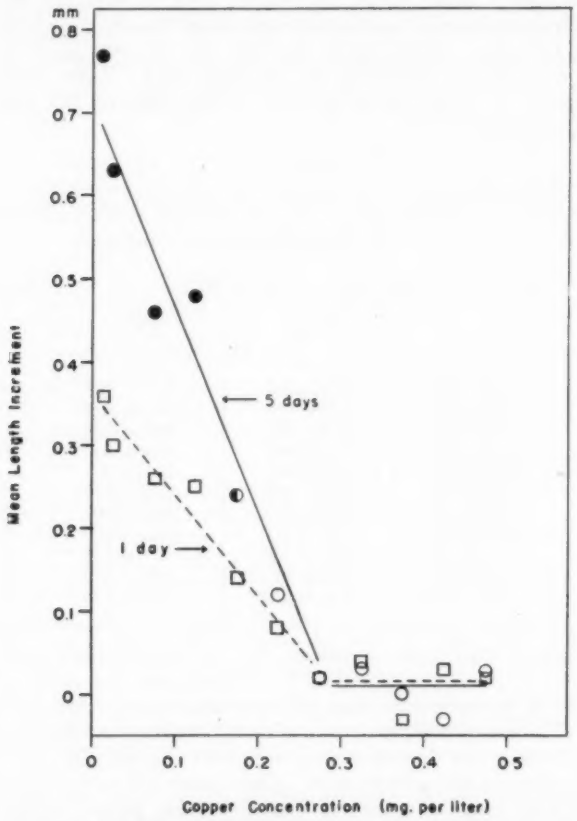

FIGURE 4. Growth of *Bugula* ancestrulae in relation to copper concentration. Curves for one-day (squares) and five-day increments (circles) based on grouped data (class intervals = 0.05 mg. copper per liter). Filled squares indicate one or more polypides per colony, half filled squares show that some stalks did not differentiate polypides, and open data points indicate that no polypides were developed. See also Figure 5.

The copper concentrations of each solution were determined before and after the experiment, and the average values were used.

As clearly shown in Table II and Figures 4 and 5, the degree of early growth and differentiation of *Bugula* is closely related to the copper concentration in the water surrounding the organisms. Up to about 0.3 mg. per liter, increment in

length is inversely proportional to copper concentration of the solution. Higher concentrations inhibit growth completely or allow but slight, insignificant increment. The sharp break or inflection in the curves in Figures 4 and 5 at about 0.3 mg. copper per liter apparently marks this as a critical concentration for growth. No stimulating effect of small amounts of copper on growth was noted, since even in the greatest dilutions used growth increments were less (with minor exceptions) than in fresh sea-water controls.

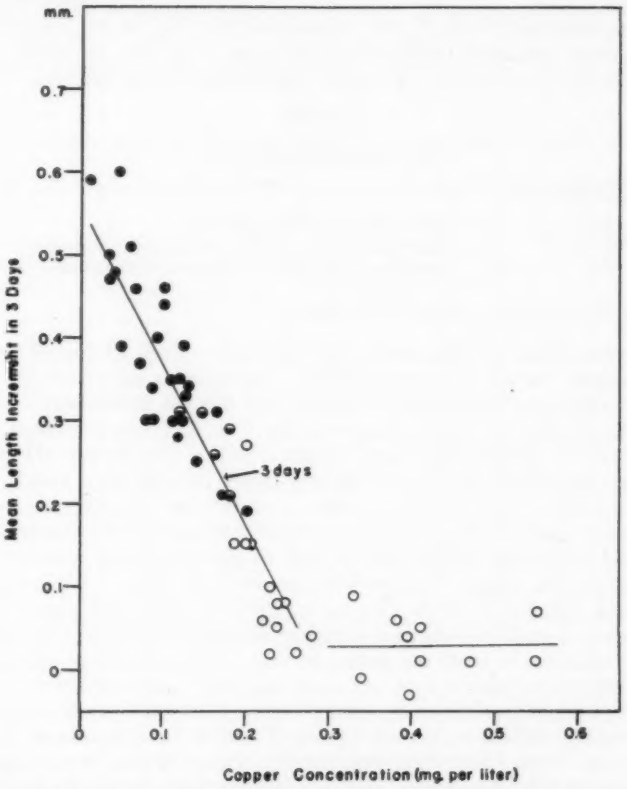

FIGURE 5. Three day growth of *Bugula* ancestrulae in relation to copper concentration. Black circles indicate fully developed polypides, half filled data points represent partly formed polypides, and clear circles indicate no differentiation of polypides. See also Figure 4.

Polypide differentiation is inhibited by copper concentrations greater than 0.2 mg. per liter, the minimal value being less than that required to prevent growth completely. Concentrations less than 0.2 mg. per liter only delay polypide development. This is shown by the fact that only controls attained the second polypide stage at three days (Table II), by the several cases of incomplete or nonfunctional polypides at three and five days in the range between 0.1 and 0.2 mg.

per liter (Figs. 4 and 5), and by the fact that at five days only the first polypide was developed in concentrations greater than 0.1 mg. per liter.

A series of critical copper concentrations may be postulated from these and other studies (Table III). The minimum lethal dose (MLD) for free swimming larvae is about 0.3 mg. per liter (Miller and Cupp, 1942), the same concentration required to stop growth of attached larvae. No data are available on concentrations necessary to prevent larval attachment. Presumably these would be at least as great as the minimum lethal dose, and probably greater, since larvae in solutions containing as much as 0.4 to 0.5 mg. copper per liter do not die immediately but swim about more and more slowly for a half hour to an hour or more. Given a suitable surface, it is conceivable that some might attach in this interval.

TABLE III

Critical copper concentrations

Copper concentration (mg. per liter)	Physiological effect
>0.3	Kills larvae. Inhibits growth.
0.2–0.3	Retards growth. Inhibits polypide development.
<0.2	Retards growth and development of polypides.

Recovery of Bugula from copper poisoning

The question arises, are the above-described toxic effects of copper permanent, or can organisms recover to any significant degree after exposures to sublethal dosages? Preliminary experiments showed that *Bugula* ancestrulae can recover nearly normal development after immersion for 6 to 24 hours in sublethal copper sea-water solutions (Miller and Cupp, 1942). In this experiment, the periods of immersion in copper solutions were extended (three to seven days) and alternated with periods of immersion in fresh sea water to determine the effect on recovery of longer exposures and of intermittent as compared to continuous dosages.

The initial procedure was similar to that of preceding experiments. A non-toxic test panel with attached larvae was immersed in each of eight copper sea-water solutions (Nos. 1–8, Table IV) for a period of three days (time period I). The subsequent procedure features a staggered schedule of transfers of the animals from copper solutions to fresh sea water, so that, while one group was exposed to copper, the other was immersed in untreated sea water and *vice versa*. After the first three days exposure, half of the panels (group *A*, sets 1–4) were transferred to fresh sea water, and the remainder (group *B*, sets 5–8) were left in the original copper solutions. After four days (period II), group *A* panels were transferred from sea water back to the original copper solutions, while group *B* sets (that had now been in the toxic solutions for a week) were transferred to fresh sea water. After another four days (period III), the process was again reversed—those in sea water were transferred back to copper solutions and *vice versa*. The experiment was terminated on the fifteenth day, the final four days constituting period IV.

Length measurements were made before the original immersion (a few hours after attachment of the larvae), at the time of transfers (on the 3d, 7th, and 11th days), and at the termination of the test on the 15th day. From these data, the rates of growth relative to that of controls for the four time periods in copper solutions and sea water were computed. Concentrations of the copper solutions

were estimated from their biological effects (using curves in Figs. 4 and 5) as chemical determinations were not made for this test. Data are given in Table IV, and the salient features are shown in Figure 6.

In all but one case, transfers to fresh sea water were followed by marked and significant increases in growth rate and polypide differentiation, while the reciprocal transfer resulted in sharply decreased, if not completely inhibited, growth (Fig. 6). The exceptional case (set 7) presumably received a lethal dose in the first period as no significant increases in length and certainly no differentiation were observed following transfer to fresh water. All others, however, exhibited an amazingly high degree of plasticity and regulatory ability in recovering from the effects of the

TABLE IV

Growth and development of Bugula ancestrulae during and after immersions in copper sea-water solutions

Symbol No.	Cu. in solns. mg./liter	Mean length increments, time periods I–IV *				Total no. of polypides per colony at end of periods:			
		I (3 days) mm.	II (3–7 days) mm.	III (7–11 days) mm.	IV (11–15 days) mm.	I	II	III	IV
A-1	0.26	0.05	0.34	0	0.14	0	1	1	2
-2	0.27	0.04	0.22	0.01	0.09	0	1	1	2
-3	0.24	0.09	0.27	0.10	0.14	0	1	1	2
-4	0.23	0.11	0.25	0.09	0.06	0	1	1	2
B-5	0.25	0.08	0.01	0.12	−0.02	0	0	1 ab.	1
-6	0.23	0.10	0.01	0.17	−0.02	0	0	1 ab.	1
-7	0.3	0.03	0.03	0.05	0	0	0	0	0
-8	0.20(?)	0.14	0.16	0.23	0.10	1	1	1	2
Control		0.68 (0.68)	0.64 (0.53)	0.11 (0.28)	0.25 (0.19)	1	2	3	4

* Group *A* immersed in copper solutions during periods I and III, and in fresh sea water during periods II and IV. Group *B* immersed in copper solutions during periods, I, II, and IV, and in fresh water during period III.

poison. Even those immersed an entire week in solutions that allowed but slight growth increment and no visible differentiation (e.g., sets 5 and 6) were able to recover significantly, from growth rates of zero to as much as 60 per cent that of controls, when restored in fresh sea water. They also differentiated polypides though these were slightly abnormal in some instances. Set 8 in group *B* was apparently exposed to a weaker solution than any of the others since these individuals not only grew considerably but also developed polypides even while immersed in the copper solution. Nevertheless, significant increases and decreases in relative growth rates for this set were observed in the various periods. Seemingly, one could control rate of growth and differentiation almost at will by appropriate exposures to copper. The ability of young *Bugulae* to recover each time after repeated and long immersions in rather toxic copper solutions is truly remarkable.

In no case was recovery complete as judged either by growth rates or polypide development. Growth rates after transfers to sea water ranged roughly between a third and four-fifths of the normal (see Fig. 6), and the number of polypides developed was less than that of controls.

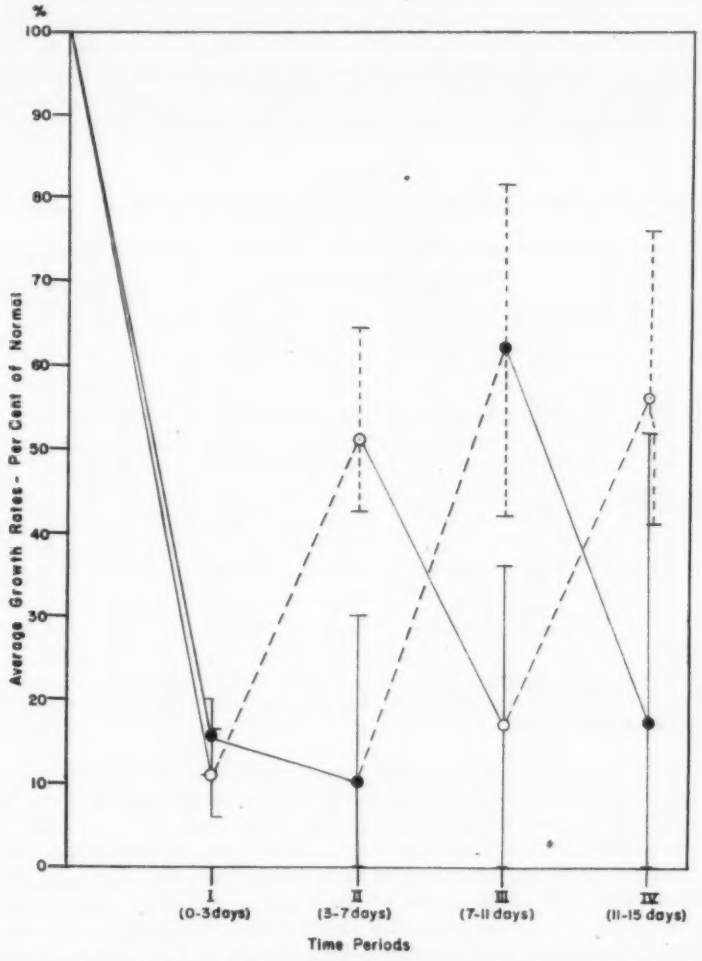

FIGURE 6. Growth rates of *Bugula* ancestrulae (in per cent of normal) during and after exposure to sublethal copper concentrations. Black data points represent averages for group *A* panels (Table IV), and clear circles are averages for group *B* panels (omitting set 7). Solid lines indicate periods of immersion in copper solutions, and dashed lines represent transfer to fresh sea water. Vertical lines through data points show the range between minimum and maximum growth rates for each average.

Length of exposure in sublethal concentrations has relatively little apparent effect on recovery. This is shown by the fact that *Bugulae* exposed to copper for seven days were able to develop after transfer to sea water at a rate comparable to that of specimens which had been exposed for only three days. As previously noted, however, the longer exposures caused abnormal polypide development in some cases.

In contrast to this finding, there is evidence that in the case of barnacles and mussels the length of exposure is as important as the concentration in determining the toxic effects of copper solutions. Clarke (1943) found that low concentrations acting for a long time produced effects equivalent to high concentration acting for a short time. In other words, the toxic effect is proportional to the product of duration and intensity. Further experiments of this general type are needed to determine more precisely immediate and long-term effects of various exposures and concentrations and of continuous versus discontinuous exposures.

These experiments have practical implications since, under natural conditions, the concentration of toxic ions adjacent to a copper paint surface might vary from time to time as a result of fluctuation in leaching rate, formation of surface films, currents flowing past the surface, and other factors. The effect of such changes on attached *Bugula* larvae might be surmised from the foregoing studies showing that growth is an inverse function of copper concentration in the medium and that stunted individuals can resume development when restored to non-toxic situations. Presumably, *Bugula* ancestrulae attached to a surface with fluctuating toxicity might recover to some degree with each significant decrease, if the organisms were still viable. With each increase in length, the ancestrulae greatly improve their chances to establish colonies for reasons which will become apparent in the next section of this paper.

Toxicity gradients of copper paint surfaces

The question of zones or gradients of toxicity adjoining toxic paint surfaces is involved in understanding their antifouling action, and is the last problem to be considered in this paper. Although a toxicity gradient has been assumed as a consequence of diffusion of toxic ions emanating from an antifouling paint, a demonstration of this seemed desirable. Furthermore, information on the effective limits and other characteristics of the toxic zone is of particular interest in connection with the establishment of colonial fouling organisms (e.g., erect bryozoans and most hydroids) that grow, plant-like, more or less perpendicularly away from the surface to which they attach, and develop new individuals at the ends of their branches. It would be useful to know how far from the toxic surface growth inhibiting and growth retarding concentrations are maintained, or how much a colony would have to grow before its terminal polypides were out of danger from poisoning. In the following experiment, the problem was to demonstrate, if possible, the existence of the toxic zone of an antifouling paint and to determine its general characteristics.

The preceding studies, showing rather precise relationships between growth of *Bugula* ancestrulae and copper concentration, suggested a method for attacking the problem of toxic gradients. The essential features of the procedure used and of the results obtained are illustrated in Figure 7. A non-toxic panel bearing at-

tached and growing *Bugulae* was placed perpendicularly against a panel coated with a cold-plastic copper-paint (Table I, No. 7). With this arrangement, the developing *Bugula* stalks maintain practically constant distances between their axes and the toxic surface since they grow parallel to the latter. The non-toxic panel was ruled in millimeter divisions paralleling the toxic surface, and these were used as class intervals of distance in analyzing the data. The ancestrulae in each division were measured at the start of the tests and two days afterwards to determine the effect of diffusing copper ions on their growth at various distances from the paint surface. For control, a non-toxic panel was substituted for the toxic panel. Experimental and control racks were placed in an aquarium containing ten liters of sea water. The water was aerated during the tests by fine streams of air bubbles delivered through pinholes in a piece of rubber tubing stretched along the bottom of the aquarium. This method of aeration caused some circulation of water but no appreciable agitation.

TABLE V

Toxicity gradients of a copper paint demonstrated by growth of Bugula at measured distances from the toxic surface

Distance from paint surface	Test 1 Mean growth increment ± S.E. (2 days)		Test 2 Mean growth increment ± S.E. (2 days)	
	Experimental	Control	Experimental	Control
mm.	mm.	mm.	mm.	mm.
0–1	0.10±.02	0.48±.02	0.01±.01	0.43±.02
1–2	0.27±.03	0.42±.05	0.03±.01	0.46±.02
2–3	0.40±.06	—	0.16±.03	0.44±.03
3–4	0.38±.06	0.50	0.30±.06	0.49±.02
4–5	0.35±.10	0.50	0.40	0.50±.04
5–6	0.45±.08	0.45±.04	0.37±.04	0.50
6–7	0.55±.04	0.45±.04	0.35	—
7–8	0.45±.08	0.40	0.45	0.40
8–9	0.50±.07	0.50	0.35±.04	0.40
9–10	0.50	0.43±.03	0.41±.02	0.50

Two tests were made: the first, using toxic panels that had been seasoned for ten weeks in the laboratory; the second, with the same panels that were seasoned another two weeks. In the first tests, the slime film which had accumulated during seasoning was not removed but for the second tests the slime was wiped off. Data are given in Table V and graphically illustrated in Figures 7 and 8.

As clearly demonstrated by the retarded growth of the colonies near the painted surface, the toxicity arising from the surface decreases rapidly with distance from the surface. The outer effective limit of the toxic zone is variable. Beyond two millimeters from the surface in the first test and four millimeters in the second, no significant difference in growth between experimentals and controls was demonstrated. These values, then, represent the respective outer limits of the toxic gradient in the two tests and indicate the order of magnitude of the width of the toxic zone for a good antifouling paint. Within these zones, growth increments are roughly proportional to perpendicular distance from the toxic surface as might be expected from the diffusion gradient of toxic ions. Ancestrulae immediately

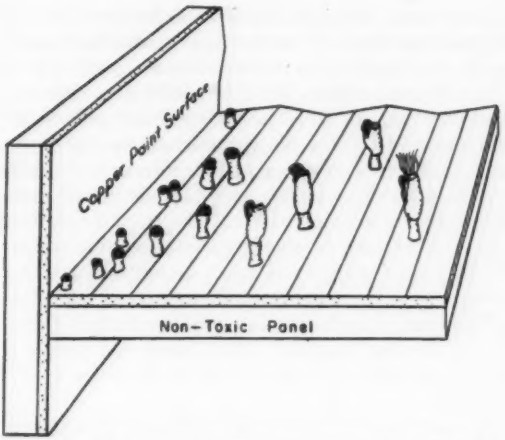

FIGURE 7. The toxic gradient extending from a copper paint surface as shown by growth of *Bugula* ancestrulae at measured distances from the toxic surface. *Bugula* figures are camera lucida drawings made four days after start of test 2 (for two-day growth, see Table V), and are twice enlarged in comparison to the millimeter rulings shown on the non-toxic panel.

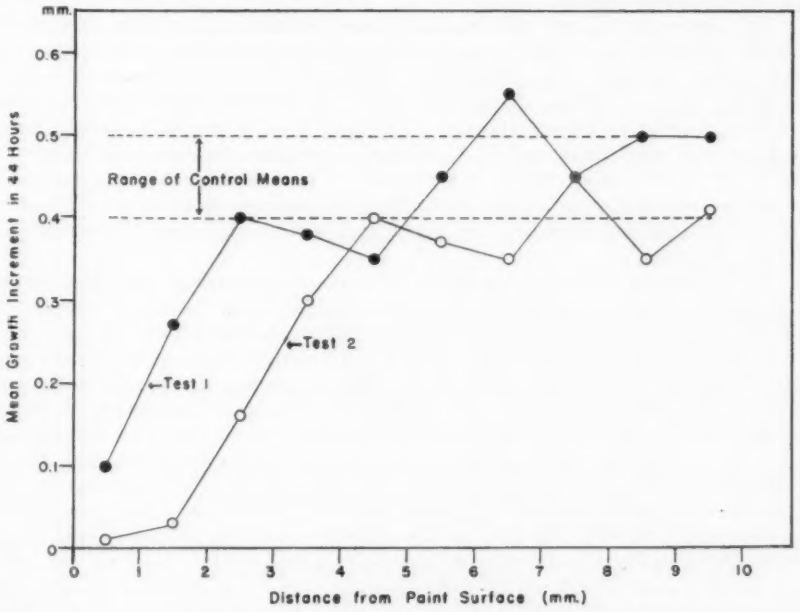

FIGURE 8. Gradients of toxicity of an antifouling paint: test 1—paint seasoned for ten weeks and coated with slime film, and test 2—after twelve weeks seasoning and with slime film removed before testing.

adjacent to the toxic paint, however, showed no increase in length indicating that growth inhibiting concentrations of copper were maintained there. This result was anticipated since larvae attached to surfaces coated with this paint do not grow (Table I, No. 7). Growth within the first millimeter interval is contributed by ancestrulae in the outer part of it. The width of the growth inhibiting portion of the toxic gradient (practically, the most important part of it) could be more precisely determined by further tests using smaller intervals of distance.

The panels used in the second test were clearly much more toxic than those first tested as shown by the greater width of the toxic zone, by the greater growth inhibition near the surface, and by the fact that the curve of growth increments at distances greater than four millimeters tends to fall below that of controls. With respect to the latter point, the mean growth increment for all individuals between four and ten millimeters in test two is significantly less than the corresponding mean in test one or that for controls. The means for single millimeter intervals in this range are not significantly different probably because of the small numbers of individuals in each.

The characteristics of the toxic zone are undoubtedly affected by various factors such as leaching rate and velocity of flow of water across the surface. In the field, currents or movement of the painted surface through the water would probably alter the character of the toxic zone. This and other factors could be simulated in the laboratory and their effects analyzed using the above-illustrated methods with appropriate modifications.

The demonstrated vertical gradient would probably differ from a horizontal gradient that extends outward from the edge of a painted surface. The latter type was nicely demonstrated by Pomerat and Weiss (1943) in field tests with panels on which areas of various shapes and sizes were left unpainted. The horizontal gradient observed by these authors was expressed both by graded growth of fouling on large unpainted areas, and by absence of fouling on smaller areas encircled by the paint. Their effects might be attributed in part to delayed larval attachment as well as retarded growth since the settling of larvae cannot be controlled in the field. For practical purposes, the activity of a vertical gradient is probably of greater importance.

The foregoing experiment together with those reported in preceding sections of this paper clearly indicates that the prevention of *Bugula* fouling on copper paint surfaces is dependent upon their ability to maintain growth inhibiting concentrations of copper in a narrow zone at the surface. The length of newly attached *Bugula neritina* larvae (about 0.2 mm.) presumably represents the minimum adequate width of the growth inhibiting zone required to prevent establishment of this organism. If attached forms are permitted to grow, their apical developing parts move away from the toxic surface and hence into regions of lower toxicity with consequent acceleration of development. As new polypides are produced by distal budding, they find themselves in a less toxic environment than their predecessors and eventually the terminal polypides would lie entirely outside of the toxic zone. Since the polypides are functionally independent (except for support), the colony can flourish even though its basal members are dead. If the toxic zone extends outward only a few millimeters, just a few of the basal polypides would be affected since *Bugula* colonies at the first polypide stage average about 0.9 mm. in length and each successive polypide adds at least a half millimeter to the length.

Larvae that settle on previously attached forms or on any inert particles elevated appreciably above the surface plane of the paint would clearly stand a better chance of survival than those attached directly to the toxic surface. They would occupy less toxic regions of the toxic gradient which might permit their growth, while those attached to the surface itself might be killed or permanently stunted by the higher concentration of the toxic prevailing there. Judging from the steep slopes of the toxicity gradients for an effective paint (Fig. 8), a fraction of a millimeter from the paint surface might make a significant difference in development, especially on surfaces with borderline toxicity.

To summarize: the foregoing preliminary tests clearly demonstrate a zone or gradient of toxicity that extends outward a few millimeters from an effective copper paint surface. Further experiments, using the method illustrated, are indicated to determine more precisely the limits and other characteristics of the toxic zone under various conditions.

Summary

Copper paint surfaces prevent the establishment of *Bugula neritina* (1) by repelling or killing the larvae and (2) by inhibiting growth and metamorphosis of attached larvae.

Copper paints with leaching rates less than 10 micrograms of copper per square centimeter per day permit the larvae to attach in large numbers and to grow and differentiate. Paints with leaching rates greater than 15 micrograms per square centimeter per day allow only a small percentage of larvae to attach and completely inhibit their growth. Large percentages of larvae occasionally attach to paint surfaces with leaching rates between 10 and 15 micrograms per square centimeter per day, but these do not develop colonies.

No consistent effect of slime film on larval attachment was noted. Its presence is not prerequisite to attachment.

Precise relationships between copper concentration and growth of *Bugula* ancestrulae are demonstrated. Growth in sea-water solutions of copper is inversely proportional to the concentration up to 0.3 mg. per liter. Higher concentrations completely inhibit growth.

The critical copper concentrations affecting various stages of the early life cycle of *Bugula* are as follows: (1) Concentrations greater than 0.3 mg. per liter kill larvae and completely inhibit growth of attached forms, (2) concentrations between 0.2 and 0.3 mg. per liter retard growth and prevent polypide formation, and (3) concentrations less than 0.2 mg. per liter retard growth and polypide development.

No stimulation of growth by copper solutions was observed. There was some evidence that small concentrations of copper stimulated attachment of larvae.

Bugula ancestrulae can recover and develop almost normally after seven days exposure to sublethal concentrations of copper. They can recover after repeated immersions in copper solutions that practically prevent growth. Length of exposure has relatively little effect on their ability to recover from copper poisoning.

A gradient of toxicity extending outward a few millimeters from a copper paint surface is demonstrated.

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A NEW GRAPHIC METHOD OF DESCRIBING THE GROWTH OF ANIMALS

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Growth curves, when conventionally plotted as length on age, are difficult to compare and classify. Moreover, the usual mathematical methods of fitting them, such as, the logistic and the Gompertz are rather laborious and inconvenient for application to large numbers of individuals.

Fortunately, for many purposes, it is unnecessary to describe the whole growth curve; for the part below the inflection point is completed early and the part above the inflection point—the "self-inhibiting" phase, covers the period of life when differences in growth are likely to be most striking. That phase of the growth curve can be appropriately represented by a straight line, the characteristics of which can be treated statistically, by the following graphic method:

Using arithmetic graph paper, with body length represented along both the x axis and along the y axis, plot length at ages 1, 2, 3, 4, $5 \cdots n$ on the x axis against length at ages 2, 3, 4, 5, $6 \cdots n + 1$, respectively, on the y axis. For several species on which I have found published length data, these points fall along a straight line. This line can be regarded as a sort of *transformation* of the usual growth curve, and in the following discussion I will call it that.

The nine examples given in Figures 1–3 are based on average lengths of large samples. When lengths of individual specimens are plotted by this method, a straight-line relationship is still obvious, though the points deviate more widely from the line than when averages are used. These deviations doubtless result from several causes, among which random variations in environmental experience and errors of observation readily suggest themselves. For a few species the published growth data failed to produce a straight line. In these cases, the course of growth may differ from that in other animals; or the observed anomalies may reflect some artifactual effect in the data.

Among those species for which this "transformation" results in a straight line, the growth increments corresponding to equal time intervals, for example, between years of age ($l_2 - l_1$, $l_3 - l_2$, $l_4 - l_3, \cdots l_n - l_{n-1}$), have the following interrelations; where l_n refers to the length at any given age, i.e., at the end of any given time interval:[2]

$$\frac{l_3 - l_2}{l_2 - l_1} = \frac{l_4 - l_3}{l_3 - l_2} = \frac{l_5 - l_4}{l_4 - l_3} \cdots = \frac{l_n - l_{n-1}}{l_{n-1} - l_{n-2}} = k$$

[1] For advice and assistance in the mathematics of this paper, I am indebted to Professors George Polya and Harold Bacon of Stanford University. I am also grateful to my colleagues, Mr. O. Elton Sette and Dr. Frances Felin, for their constant interest and help.

[2] In this discussion, l_1 is not necessarily the value directly obtained by measurement, but a value calculated on the basis of all measurements (*see* Fig. 4).

or

$$l_n - l_{n-1} = k\,(l_{n-1} - l_{n-2}). \tag{1}$$

It is interesting to note that, with $l_0 = 0$,

$$\begin{aligned}
l_2 - l_1 &= k\,(l_1 - l_0) = k\,l_1 \\
l_3 - l_2 &= k\,(l_2 - l_1) = k\,k\,l_1 = k^2\,l_1 \\
l_4 - l_3 &= k\,(l_3 - l_2) = k\,k^2\,l_1 = k^3\,l_1 \\
l_5 - l_4 &= k\,(l_4 - l_3) = k\,k^3\,l_1 = k^4\,l_1 \\
&\cdots\cdots\cdots\cdots\cdots\cdots\cdots \\
l_n - l_{n-1} &= k\,(l_{n-1} - l_{n-2}) = k\,k^{n-2}\,l_1 = k^{n-1}\,l_1.
\end{aligned} \tag{2}$$

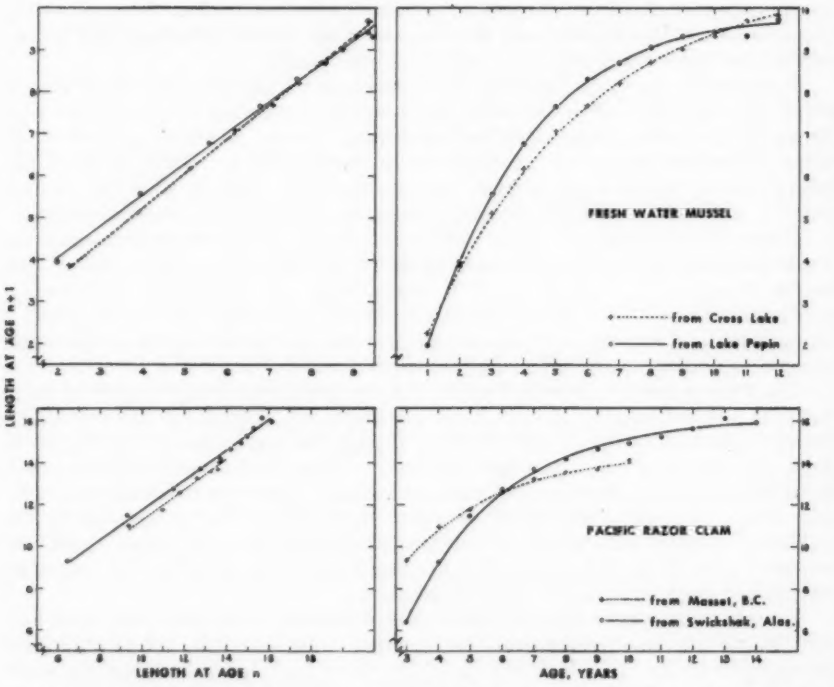

FIGURE 1. Left-hand series: growth data plotted according to the method described, and fitted empirically with straight lines. Right-hand series: the same data converted to conventional size-on-age curves. Data on fresh water mussel from Chamberlain (1931); on Pacific razor clam from Weymouth (1931).

The constant k is positive and less than one; that is, the yearly growth increments decrease.

These relationships are consistent with there being a growth capacity, which is approached from the inflection point at a constant percentage rate. This is in accord with interpretations made by a number of students of growth, among

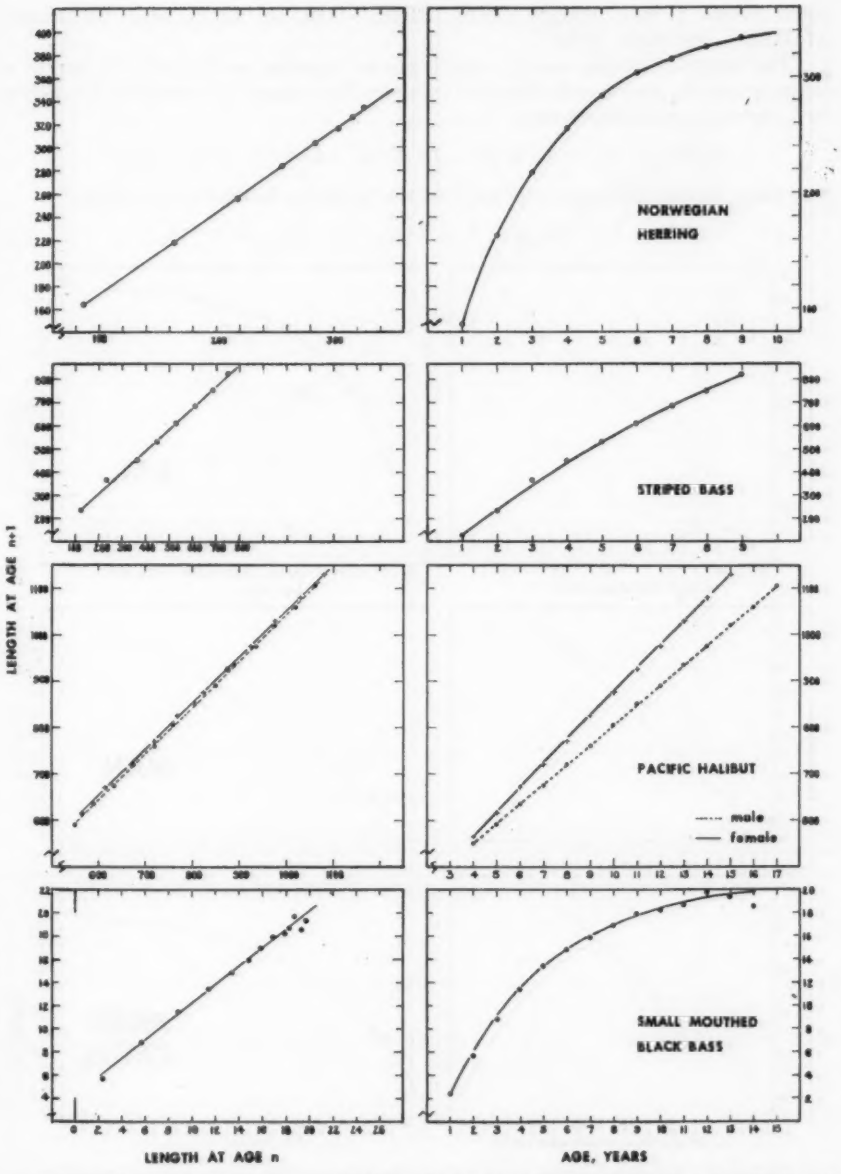

FIGURE 2. Left-hand series: growth data plotted according to the method described, and fitted empirically with straight lines. Right-hand series: the same data converted to conventional size-on-age curves. Data on Norwegian herring from Runnström (1936); on striped bass from Merriman (1941); on Pacific halibut from Thompson and Bell (1934); on small mouthed black bass from Bennett (1938).

them Minot (1908), Wright (1926), Brody (1927 a, b, c), and Weymouth, McMillan, and Rich (1931).

The length at infinite age, l_∞, which can be regarded as the ultimate length or limiting length, can be calculated as follows: The length l_n is attained by adding to l_1 the successive increments,

$$l_n = l_1 + (l_2 - l_1) + (l_3 - l_2) + (l_4 - l_3) + \cdots + (l_n - l_{n-1}).$$

Yet these increments were expressed in the formulas following (1), thus:

$$l_2 - l_1 = k\, l_1, l_3 - l_2 = k^2\, l_1, \cdots l_n - l_{n-1} = k^{n-1}\, l_n. \tag{3}$$

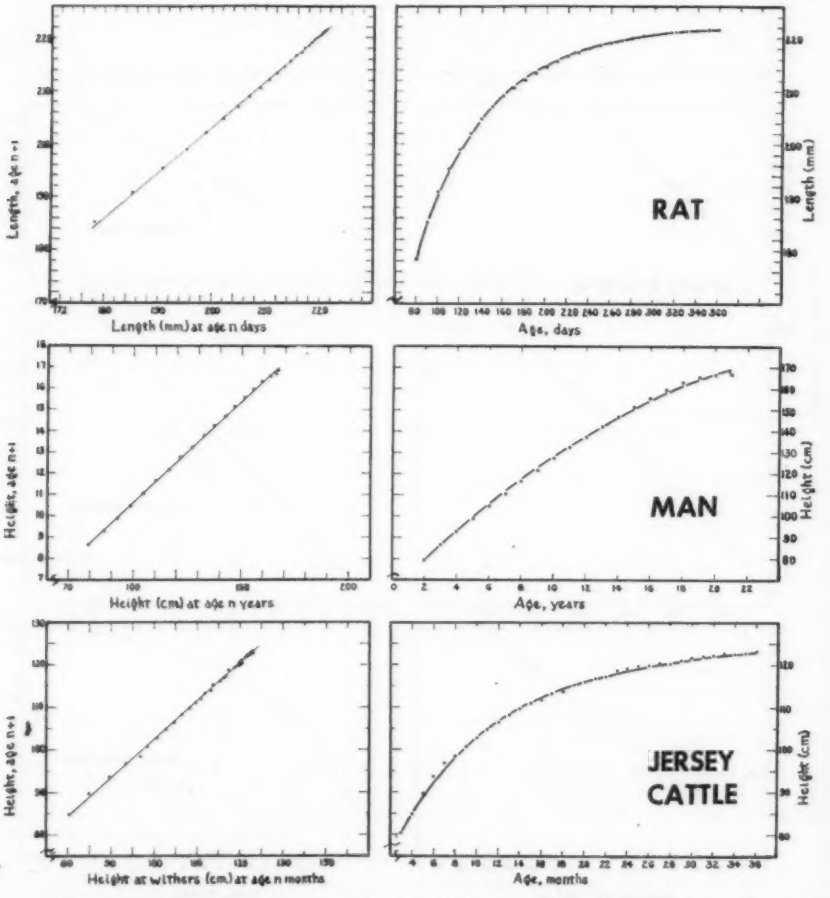

FIGURE 3. Left-hand series: growth data plotted according to the method described, and fitted empirically with straight lines. Right-hand series: the same data converted to conventional size-on-age curves. Data on rat from Donaldson (1931); on man from Thompson (1942); on Jersey cattle from Ragsdale, Elting, and Brody (1926).

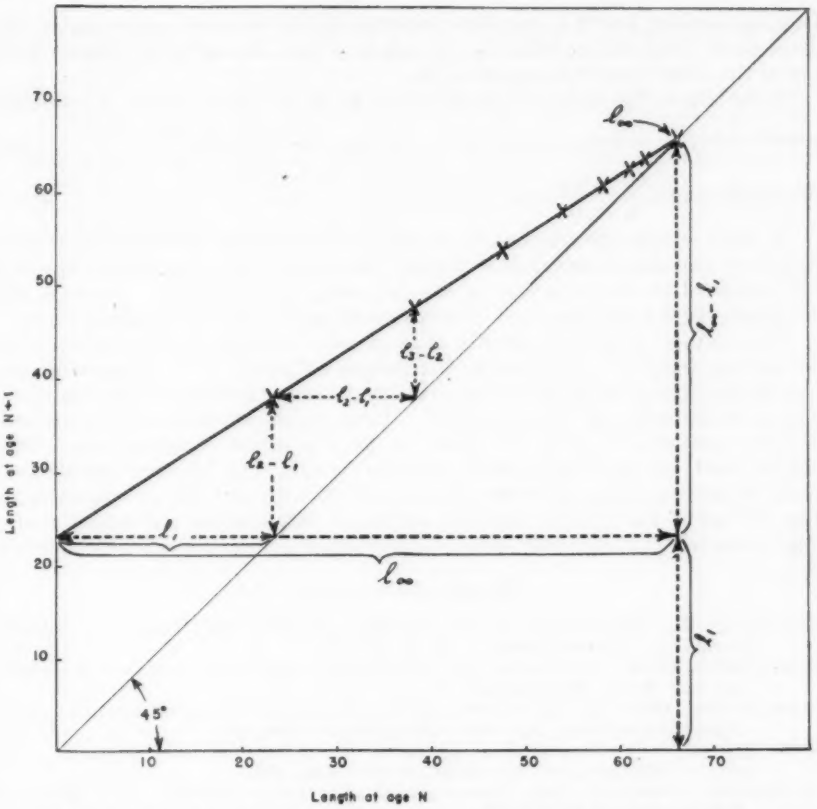

FIGURE 4. Transformation of the growth curve of a hypothetical animal, drawn in a heavy line to illustrate the dimensions given in the text. Note that the ultimate length, l_∞, can be located graphically as the point where the length at age n equals the length at age $n + 1$; also where the transformation intersects a line drawn at 45° through the zero point.

Therefore,

$$l_n = l_1 + k\,l_1 + k^2\,l_1 + k^3\,l_1 + \cdots + k^{n-1}\,l_1 = l_1\frac{1 - k^n}{1 - k} \qquad (4)$$

by the well-known formula for the geometric series. Therefore, when n approaches ∞, l_n tends to l_∞, and k^n to 0, and so we obtain

$$l_\infty = \frac{l_1}{1 - k}. \qquad (5)^3$$

[3] Where this graphical representation gives precisely a straight line, the above calculation shows that l_n is expressed by the formula $l_n = l_\infty(1 - k^n)$. It is a modification of the "Modified Exponential" (cf., Croxton and Cowden, 1940, pp. 441 ff.), but contains one less parameter, and of course applies only to that segment of the growth curve above the inflection point.

Thus the limiting length l_∞ may be computed readily from the y intercept, l_1 and the slope of the fitted straight line, k; and k, in turn, is readily calculated from any of the ratios preceding equation (1).

According to the series of equations (1) to (4) the slope of the transformed growth curve, k, is the constant given not only by the ratio $\frac{l_n - l_{n-1}}{l_{n-1} - l_{n-2}} = k$ but also by the ratio $\frac{l_\infty - l_{n+1}}{l_\infty - l_n} = k$.

In other words, the amount of growth which remains unfulfilled at the beginning of any time interval, is a constant percentage of the amount of it which had remained at the beginning of the preceding time interval. Consequently the higher the k value, the more slowly growth approaches the limiting length.

This method of plotting growth data permits an objective determination of the limiting length, l_∞, even before that length is "attained." [4] It provides two growth characteristics, k and l_∞, from which the upper segment of the length-on-time growth curve can be reproduced. These constants are so simply derived, that it is practical to determine them for large numbers of individuals. They can be used for studying growth variation within and between populations, hence for distinguishing between races of animals with differing growth patterns. The method is particularly useful in studies of fishes whose scales bear annual rings, from which the growth history of individual specimens can be estimated.

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[4] Strictly speaking, of course, under the terms of this description the limiting length is only approached; it is never attained.

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INVESTIGATION ON THE LOCUS OF ACTION OF DDT IN FLIES (DROSOPHILA)

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Introduction

The experiments reported herein were designed to gain information as to where DDT produces its poisonous effect in the insect.

DDT poisoning in insects is characterized by symptoms of hyperactivity and discoordination of neuromuscular system, followed by convulsions and terminating in death. Isolated legs of DDT treated roaches continue to twitch after complete separation from the body, and isolated legs of normal roaches were induced to twitch by the application of DDT to the cut surface, although they remained quiescent if DDT was not applied (Yeager and Munson, 1945). Likewise the isolated legs of adult blowflies (*Phormia regina*) showed twitching movements when dipped before or after they were cut off from the animal into a one per cent DDT acetone solution, while untreated isolated legs remained motionless (Chadwick, 1945). From somewhat different experiments, Tobias *et al.* (1945) working with roaches suggested that the thoracic ganglia were the critical loci for the action of DDT. From these observations it appeared likely that the symptoms of DDT poisoning in insects resulted from an effect on the central nervous system, but that there existed also peripheral components which were not as yet exactly delimited.

Material and Methods

The experiments were performed on the larvae and adults of the fruit-fly (*Drosophila virilis*). The DDT preparation used was in form of an emulsion, (one per cent DDT, one per cent lecithin, ten per cent peanut oil, emulsified in a 0.95 per cent NaCl solution). This emulsion was injected by means of a micropipet into the abdominal cavity of the insect. The physiological saline solution used throughout the investigation was a Ringer solution modified for Drosophila (H_2O, 1000 cc.; NaCl, 7.5 gm.; KCl, 0.35 gm.; $CaCl_2$, 0.21 gm.). The various concentrations of phenobarbital used were also always made up in this Ringer solution. Imaginal discs were transplanted with the usual Drosophila transplantation technique.

Experimental

Behavior of larvae and adults after poisoning

When DDT emulsion was injected into the abdomen of adult flies which had been slightly narcotized with ether, the response to the poison was immediate. Legs and wings at once went into violent, uncoordinated movements. About twenty seconds after the injection the abdomen, previously motionless, began to

convulse; its movements were a rapid succession of short, uncoordinated spasmodic twitches. At first the convulsions were strong and a great deal of the injected emulsion was thus pressed out through the puncture wound. About five minutes after the injection the legs and wings went into a spasm and took up a characteristic position. The legs were drawn toward the body and crossed over ventrally, while the wings were folded backward. This position was maintained until the animal died. The contractions of the abdomen continued for about four to seven hours but became gradually weaker. After seven hours the animal was apparently dead. Although during all of this time the legs and wings remained in their spasmodic condition, one occasionally observed slight twitches of the tarsal segments and of the antennae; the wings, however, showed no movement. It was thus clear that the muscular response to the poison varied in different regions of the body, for the wing and leg muscles soon went into contraction and remained that way, while the muscles of the abdominal wall continued to convulse for a long period.

When an emulsion prepared in the same manner, but containing no DDT, was injected into flies, no effect was noted. The animals recovered from narcosis in the usual way, and were still alive the following day without any apparent injurious effects. Thus the symptoms described above were due to DDT and were not caused by the emulsion itself.

Larvae narcotized with ether were motionless, except for the pulsating heart tube visible through the transparent skin. When such larvae were injected with DDT emulsion, one observed at first a great acceleration of the heart-beat. Convulsions of the body wall began about twenty seconds after the injection. It was difficult to observe the heart-beat while the convulsions were in progress, but it was found in ligatured larvae, which will be described below, that the heart-beat soon became normal again after the initial acceleration. The larval convulsions were very strong and uncoordinated. Contraction wave after contraction wave passed over the creature, somewhat resembling crawling movements, yet the animal was unable to move from its place. The forward and backward movements were much more rapid than the normal crawling movements. Moreover, the animal never extended to its full length but remained partly contracted all the time. Short twitching contractions occurred in various parts of the body, and broke the wave-like contraction into a complex, uncoordinated movement. The larvae moved continuously in this way, some of them for twenty or more hours. The majority of such larvae died within ten hours, but some of them lived for twenty-thirty hours after the injection. The symptoms were the same, whether last (3rd) or late 2nd instar larvae were used for the experiments.

In control experiments, where emulsion containing no DDT was injected, no such symptoms occurred.

Ether Narcosis and DDT Symptoms

Normal flies etherized only until their movements stopped were completely relaxed if removed at that time from the ether. Their wings were in normal resting position and their legs were bent in the way assumed by flies at rest. Such flies recovered about one half hour after their removal from the anaesthesia and then seemingly behaved normally. Yet flies left for a longer time in the etherizer behaved quite differently. Their wings were folded back and their legs were

stretched out and held stiffly away from the body. Usually flies thus over-etherized did not recover from the narcosis and died in that position.

The reaction of these differently etherized flies to DDT poisoning was quite interesting. The slightly etherized individuals showed, after DDT injection, the typical DDT symptoms described in the foregoing section. On the other hand, when over-narcotized flies were injected with DDT, one noticed that their legs and

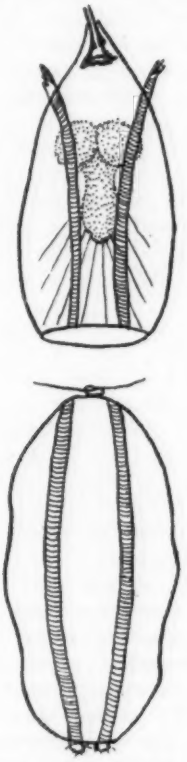

FIGURE 1. Diagram illustrating larval ligature experiments for separating the central nervous system from the rear part of the body. Note location of central nervous system (stippled) in anterior part.

wings did not respond to the poison. Since such flies never recovered from the narcosis it might be thought that they were already dead at the time of the DDT injection. This, however, was not the case, for their abdomens showed the typical DDT convulsion. These convulsions continued for about three hours, but were somewhat weaker than those of the slightly etherized animals. About that time the uninjected but over-etherized control animals were still completely immobile and apparently dead. In comparing the uninjected and injected flies, one gained the impression that the DDT treatment in some way partly released the ether block.

The Importance of the Central Nervous System

The central nervous system of Drosophila is concentrated in the anterior part of the body. In the larva the central nervous system is located in the third thoracic and the first abdominal segments; it consists of the two brain hemispheres, to which is attached a large ganglionic mass (Figs. 1 and 3). This large ganglion is a compound structure, for it includes the sub-oesophageal ganglion, the three thoracic ganglia, and the eight abdominal ganglia. In the adult insect the three thoracic ganglia are separated, but all eight abdominal ganglia unite into one ganglionic mass which extends into the first segment of the abdomen (Fig. 2).

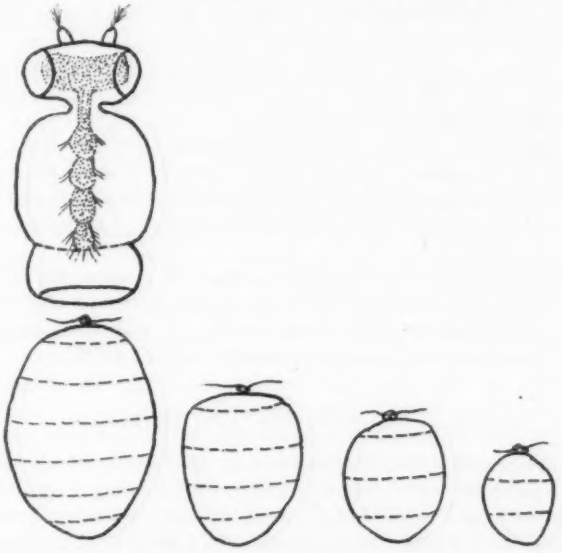

Figure 2. Diagram illustrating ligature experiments on adult flies, showing the separation of progressively smaller abdominal parts from the anterior region of the animal. Note location of central nervous system in anterior part of fly.

The described topography allowed one to separate experimentally large parts of the insect body from its central nervous system. The separation was accomplished by means of a fine silk ligature tied around the body of the animal. Depending upon the position of the ligature, smaller or larger parts of larvae and adults were thus isolated. In all experiments of this kind, the part in front of the ligature was cut away, in order to be sure that no connection to the ganglia remained (Figs. 1 and 2). Abdominal parts isolated in this way were completely motionless and stayed alive for several days.

The most useful symptom for the experimental approach was the contraction of the abdominal wall muscles under the influence of DDT. This effect was very uniform, clearly observable, and lasted a considerable time. The question then arose: were these movements under the control of the central nervous system?

In other words, was the muscular activity caused by an effect of DDT on the central nervous system? To test this possibility, isolated portions of larval and adult abdomens containing no ganglia were injected with DDT emulsion. The response to the injection was called forth immediately. Both larval as well as adult abdomens exhibited the same typical movements that were observed in the abdomens of injected intact flies. Again, as in the intact animals, it was found that the isolated adult abdomen responded somewhat faster than the isolated larval abdomen. That is, the larval abdomen responded at about thirty-sixty seconds and the adult abdomen about twenty seconds after the injection. The DDT convulsions of the isolated parts continued for a considerable time, but not as long as in the intact animals. In the isolated adult abdomen the convulsions clearly became weaker two hours after injection, yet weak contractions were observed five hours after the injection. In the isolated larval abdomens the contractions were still strong four hours after the injection, yet these parts never survived for twelve hours as injected whole larvae commonly did.

A similar sequence of events was observed when only the distal two or three segments of the adult abdomen were isolated from the rest of the body and then injected with DDT emulsion (Fig. 2) or when only two or three segments from the middle of the larval abdomen were isolated by means of two ligatures and then injected.

These findings showed that the central nervous system did not control the abdominal symptoms produced by DDT, since they occurred also in the absence of the central nervous system. However, it must be recognized that this does not imply that the central nervous system was unaffected by DDT.

Body Fragments and DDT Symptoms

In order to reduce the structural complexity of the insect body, in an effort to localize more closely the site of action of DDT, the following fragmentation experiments were performed. Rectangular pieces of skin were cut from the dorsal, lateral, or ventral wall of adult flies and placed in a drop of saline solution. These pieces, about two segments wide, included some muscles of the body wall, fat tissue, tracheae, and nerves but no ganglia. The pieces of dorsal body wall included also part of the heart tube and some alary muscles. In saline solution these pieces remained motionless, but if a few drops of DDT emulsion were added, the isolated muscles in the piece began to twitch. This response began about thirty seconds after DDT was added and continued for about two or three minutes. In control experiments where emulsion containing no DDT was added, no such response occurred.

These experiments confirmed those above by showing that the central nervous system was not necessary for the response of the abdominal muscles to DDT. It would appear that DDT affected either the muscles directly or the peripheral nerves. One way of distinguishing between these two possibilities would be to test a nerve-free muscle preparation with DDT. Technically, however, it is impossible to obtain such a preparation. Other methods were sought to settle this question and are described below.

Treatment With Phenobarbital

Phenobarbital is a drug which depresses central nervous activity in vertebrates. The effect of this substance on flies has not been described previously. When a ten per cent phenobarbital saline solution was injected into the abdomen of adult flies the animals were apparently killed instantly. The slight vibrating movements characteristic of lightly etherized flies stopped immediately after the injection. All of the muscles seemed to relax and the legs and wings were held in normal position.

Flies which had just come out of ether narcosis but were still sluggish lost their coordination when injected with a one per cent phenobarbital solution. Wings, legs, and abdomen moved uncoordinatedly for several hours until the animal finally died. The movement of the abdomen, also uncoordinated, was very different from the symptoms produced by DDT.

Injection of 0.1 per cent phenobarbital solution into adult flies induced narcosis, followed by complete recovery of the animal. One hour after the injection some flies crawled about slowly, while others were still unable to hold themselves up and fell over from time to time. By this time the effect of the ether had worn off and these symptoms were regarded as phenobarbital effects. One-half hour later the coordination of the animals had improved but their movements were still slow. But the next day the animals had recovered and behaved normally.

That phenobarbital affects the nerves rather than the muscles in flies was indicated by the following experiments. Fully grown larvae were split open along the mid-dorsal line. The intestine, Malipighian tubes, fat body and the main tracheae were then removed. Care was taken not to disturb the brain and ganglia in the anterior part of the body. This manipulation was carried out in physiological salt solution. The skin with its muscular layer and the adhering nervous system was placed in a small dish with a wax bottom and covered with fresh physiological salt solution. The preparation was then stretched out and by means of fine pins was fastened to the wax bottom of the culture dish, as shown in Figure 3. In this condition the tissues stayed alive for several hours. From time to time the muscles contracted, showing the typical wave-like contraction pattern of a crawling larva. After these movements had been observed for ten minutes the physiological solution was removed and replaced by a one per cent phenobarbital solution. The movements in the preparation stopped immediately. Again, after about ten minutes during which time no movements occurred the phenobarbital solution was removed and replaced by physiological saline. This solution was in quick succession drawn off and replaced about two or three times. The preparation was thus washed clean of any phenobarbital. It was now observed that normal movements had returned.

Ten minutes later the physiological solution was removed and replaced by one per cent phenobarbital solution; all movements again stopped. When washed and placed in physiological solution again the movements in the preparation reappeared. This procedure was repeated three or four times at intervals of about ten minutes and still the tissues continued to contract spontaneously when in physiological solution.

These experiments indicate that the one per cent phenobarbital solution was apparently not injurious to the tissues for the length of exposure tested. Moreover, while in phenobarbital, no muscular movement was observed, yet if the

muscles were stimulated directly by touching them with a fine needle, localized contraction in the region of the stimulus was noted. Following the mechanical stimulus the activated muscles contracted rather rapidly, stayed in the contracted state for some time and relaxed very slowly. This localized response of the muscles to mechanical stimuli when under phenobarbital indicates that the drug had not paralyzed the muscles directly but rather the nerves.

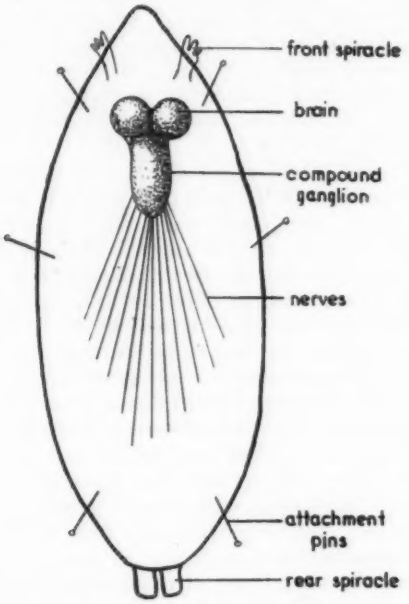

FIGURE 3. Diagrammatic representation of the skin muscle preparation of a whole larva, pinned to the wax bottom of the culture dish.

COMBINED PHENOBARBITAL AND DDT TREATMENT

The knowledge that phenobarbital apparently acted on the nerves but not on the muscles provided a tool for determining whether DDT affects the nervous tissue or the muscles. If DDT could affect the muscles directly DDT symptoms should be provoked in animals paralyzed by phenobarbital. If, on the other hand, DDT affects only the nerves, no DDT symptoms should occur in animals treated with phenobarbital. That the latter is the case was shown by the following experiments.

In physiological solution the muscle preparation (Fig. 3) showed spontaneous crawling movements as described above. If a small amount of DDT emulsion was dropped onto such a preparation, the rhythm of the movements was interrupted; it became uncoordinated rapidly and resembled the movements observed in DDT-treated larval abdomens. If, on the other hand, DDT emulsion was dropped in the same manner onto preparations kept in one per cent phenobarbital solution, no

movement whatsoever took place. Moreover, if the physiological solution containing DDT emulsion was washed off from the actively moving preparation, and was replaced by one per cent phenobarbital solution, the movements ceased immediately. However, it was impossible to bring the preparation once treated with DDT back to its normal way of movement by washing the DDT solution off and replacing it with pure physiological solution. Such preparations still showed DDT symptoms. Apparently it was impossible to wash all the DDT out by the methods used. The DDT symptoms were of course stopped by placing the preparation again into phenobarbital.

Similar results were obtained when DDT-treated larval or adult abdominal parts were injected with one per cent phenobarbital solution. The DDT symptoms of such parts ceased immediately after phenobarbital was administered by injection. The reciprocal experiment, where phenobarbital was injected first, yielded the same results, for when DDT was injected into such treated abdomens no DDT symptoms occurred. The symptoms of whole larvae or adults which had been treated with DDT were also stopped immediately by injecting a one per cent phenobarbital solution.

Certainly the effects of phenobarbital on isolated, DDT-treated abdomens indicate that the absence of the central nervous system did not limit the action of the drug, showing that in insects phenobarbital may act on the peripheral nerves.

At this point the discussion of the phenobarbital effects on DDT poisoning must be augmented by an experiment showing how phenobarbital in weaker concentrations caused at least a partial recovery from DDT poisoning. It has been stated before that the legs and wings of adult flies injected with DDT were completely paralyzed five minutes after the injection. Now, when 0.1 per cent phenobarbital solution was injected into such animals, the movements of their legs were restored. These movements were uncoordinated and were similar to those observed in the beginning stages of DDT poisoning before the organism went into spasm. Also, the wings were able to move somewhat and were not folded backward. Even the convulsions of the abdomen were much less pronounced. Leg movements continued for about two hours, which of course was a much longer time than ever noted in animals injected with DDT only. These findings clearly show the antagonistic effect of phenobarbital on DDT.

Capacity of Different Tissues for Growth and Differentiation After DDT Poisoning

If it is true, as the experiments seemed to indicate, that DDT affects only the nervous system, one might expect other tissues to be largely unharmed by the DDT treatment. This expectation can be tested experimentally. It is known (Bodenstein, 1943) that larval tissues will grow and differentiate normally when transplanted into the abdomen of larval or adult flies. In the larvae the transplanted tissues will develop in synchrony with their hosts to imaginal completion, and in adult flies, the transplant will undergo a considerable amount of growth. The capacity of the tissue for growth and differentiation, it is believed, offers a good criterion for testing the condition the tissue is in after being exposed to the poison. Tissues affected by DDT should certainly not develop normally. The following experiments were designed to clarify this issue.

Fifteen last-instar larvae were injected with DDT and placed on moist filter paper. Twenty-two hours later four larvae were still alive and showed typical DDT symptoms. Three of these larvae were opened and their eye discs, leg discs, and antennal discs dissected out. These discs are the primordia for the future eye, leg, and antenna of the adult fly.

They were transplanted into the abdomen of the mature larvae, one disc to each host. The several hosts carrying the transplants from one donor were kept separated from the hosts carrying the transplants of the other donors.

If the transplanted organs were able to develop normally, they should have been found as fully differentiated organs in the abdominal cavity of their respective hosts after metamorphosis. From the fifteen original hosts comprising the cases of all three series, eleven survived the operation. These animals completed their metamorphosis seven days after the operation and emerged. They were then dissected. Three completely differentiated legs, one eye, and one antenna supplied by the first donor were recovered. Hosts which received transplants from the second donor yielded three legs, two antennae, and two eyes, and from the third donor, three legs. All transplants were fully differentiated. As far as the detailed morphological differentiation of the tested organs was concerned, they were found to be completely normal, and there was no reason to believe that the histological differentiation likewise was not normal.

For another experimental series, five leg discs, dissected from the fourth living larva of the original set of DDT-treated animals, were transplanted into the abdomen of five adult flies. Each host in addition to the leg disc also received two ring glands. This structure is necessary for the continued growth of the transplant, for it furnishes a growth-promoting hormone (Bodenstein, 1943). Three days after the operation one host was killed and the leg disc dissected. It had grown but little. Three other hosts were killed six days after the operation and the transplants dissected. These leg discs had clearly become larger. Finally the last host was killed 24 days after the operation and the disc dissected. In this case the transplant had grown considerably and had reached an advanced state of differentiation. The results of this series of experiments were very similar to those obtained in transplanting normal discs in the same manner (Bodenstein, 1939 and 1943).

In conclusion, these two experimental series show that exposure to DDT for twenty hours in no way affected the capacity of the imaginal tissues for growth and differentiation. Hence these findings are further evidence that the nervous system alone is affected by DDT.

Summary

1. The larvae and adults of *Drosophilla virilis* were fatally poisoned by injecting a one per cent DDT emulsion into the abdominal cavity. The poison produced a typical pattern of symptoms.

2. The neuromuscular system of the wings and legs was apparently very sensitive to the poison, for they went into spasm long before the muscles of the abdominal wall. There was also a difference in sensitivity to the poison between the larva and adult, the larva being more resistant to the DDT emulsion.

3. Phenobarbital was found to affect the nervous system. Paralysis by phenobarbital was also produced in the absence of the central ganglia. This shows that the drug also affected the peripheral nerves. Muscles of larvae treated with phenobarbital responded to mechanical stimulation.

4. Since DDT produced no symptoms in animals treated with phenobarbital and since animals treated with DDT lost their DDT symptoms when injected with phenobarbital, it was shown that DDT acted on the nervous system. Moreover, body parts which had been isolated from the central nervous system and then treated with DDT stopped convulsing after phenobarbital administration. This shows that DDT affected the peripheral nerves.

5. The methods used do not allow one to determine what part of the peripheral nervous system might be affected. There are three possibilities. The poison might affect (1) the motor nerves leading to the periphery; (2) the myoneural junctions; (3) the peripheral nerve net. It is however still questionable whether such a nerve net exists in *Drosophila*.

6. The antagonistic effect of phenobarbital on DDT was clearly indicated by the fact that the spasm of the legs and wings in DDT-treated flies was partly relieved by treatment with phenobarbital.

7. The conception that only the nervous system is affected by DDT has been strengthened by the fact that larval organs (imaginal discs) which had been exposed to DDT for twenty hours grew and differentiated normally when transplanted into untreated larvae.

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THE CONTRACTILE VACUOLE AND THE ADJUSTMENT TO CHANGING CONCENTRATION IN FRESH WATER AMOEBAE

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Introduction

In considering the relationship existing between an organism and its environment, it is necessary to distinguish clearly between the environment of the organism and the environment of the individual cells. The effect of the environment on an organism will depend specifically on the extent to which the cellular environment is controlled by the organism as a whole. As pointed out by Krogh (1939), the osmotic environment of the tissue cells of the vertebrates—the body fluid—is maintained at a very constant level. This is true to a lesser degree among the lower organisms. In the free-living protozoa, the cellular environment is the outside environment of the organism and is controlled little, if at all, by the organism. We find consequently that the individual cells of higher organisms have lost their resistance to osmotic changes while the cells of many of the protozoa, of necessity, have retained the ability to withstand considerable variation in the osmotic environment. It is well known that certain bacteria and molds can withstand enormous osmotic changes—which also have been shown to be true of a considerable number of protozoa (Hopkins, 1938; Butts, 1935; Mast and Hopkins, 1941; and Kitching, 1938).

The nature of the adjustments made by cells capable of withstanding great osmotic changes is not clearly understood. In the literature, the assumption is rather generally made that the osmotic concentration of the cellular contents must be maintained at a fairly constant value in order to be consistent with the metabolic processes. This assumption is made, regardless of the fact that evidence for the constancy of the intracellular osmotic pressure is conspicuously absent, especially for the lower organisms. We find that the prevailing opinion as to the function of the contractile vacuole of the protozoa is that it is an organelle which operates to maintain the intracellular osmotic pressure at a relatively constant level which is higher than that of the environment. This theory maintains that excess water, diffusing through the outer membrane into the hypertonic cell, is extracted, isolated into the vacuole, and thus is eliminated when the vacuole is discharged (Kitching, 1938). However, Mast and Hopkins (1941) have shown that the marine *Amoeba mira* (*Flabellula mira* Schaeffer), which can withstand enormous changes in the

[1] I am greatly indebted to the American Philosophical Society for grants which have made this investigation possible. I am also much indebted to the following students of Mundelein College for valuable technical assistance: Collette Bergeron, Winifred Greenspahn, Dorothy Homan, Marie Kioebge, and Catherine Miller. To Kathleen Warner, Instructor in Biology at Mundelein College, is due grateful acknowledgment for help in the preparation of the manuscript.

concentrations of the salts of sea water, does not maintain a constant intracellular osmotic pressure. The intracellular pressure varies with that of the environment. They have shown that, if the amoeba is living in, and is adjusted to a medium, the intracellular pressure is always only slightly higher than that of the environment. Thus, a change in the osmotic pressure of the environment results in a corresponding change in the intracellular pressure.

In view of the fact that *Amoeba mira* is a marine organism and does not form contractile vacuoles even in dilute media, it was considered important to extend the investigations to a fresh water amoeba, which forms and eliminates contractile vacuoles, in order to ascertain whether these forms actually maintain a constant osmotic pressure by means of the action of the contractile vacuoles. The amoeba selected was a fresh water form collected from a swamp near Chicago. A single amoeba was isolated and allowed to multiply on an agar culture, using the methods of Rice (1935). All experiments were performed on the progeny of this amoeba.

Methods and Results

Description of the amoeba

As in the case of most amoebae, it is difficult to decide upon its correct name. Figure 1 is a composite diagram illustrating its structure as observed in fresh water. Dujardin's *Amoeba lacerata* (1841) fits it fairly well. We shall then adopt the name of *Amoeba lacerata* Dujardin. Schaeffer (1926) has placed amoebae of this type in the genus *Mayorella* Schaeffer. Thus, using the system of Schaeffer, the name is *Mayorella lacerata* Dujardin.

Amoeba lacerata is a small amoeba, rounds up readily when disturbed, and in that condition has an average diameter of fifteen micra or a volume of about 1500 cubic micra. It varies from a limacine to a flattened fan shape and has a clear hyaline border (Fig. 1*a*) which is thick anteriorly and very thin posteriorly. A few, fine, pointed, indeterminate pseudopodia may project from the surface—anteriorly, superiorly, and posteriorly. Cytoplasmic granules are numerous—(1) an abundance of small, barely visible granules and (2) larger granules (or vacuoles) which are less numerous and more variable in number and size (Fig. 1*b*). The living nucleus (Fig. 1*d*) is spherical with a central endosome. Food is engulfed into the under surface as the amoeba moves over the food particles. Except in rare cases, food is engulfed with little or no water. Ingested food particles coalesce with clear vacuoles (Fig. 1*e*) of the cytoplasm, and the resulting small food vacuoles coalesce with each other as they do in *Amoeba mira* (Hopkins, 1938) to form large food vacuoles (Fig. 1*f*). These vacuoles are readily stained with Nile blue sulfate and neutral red. Egestion of food vacuoles (Fig. 1*g*) occurs at the posterior tip.

No direct connection or relationship between the food vacuoles and the contractile vacuoles has been observed except that occasionally a food vacuole may be ruptured into the contractile vacuole instead of to the outside. At egestion, the food vacuoles are rarely large and watery. Just before egestion they may be observed to decrease slowly in size until little more than food residues remain. This would indicate a protoplasmic absorption of water from the food vacuoles. It is also interesting to note that at the time when this apparent absorption takes place, the food vacuoles are located posteriorly and in close proximity to the region of

contractile vacuole formation which MacLennan (1941) and others have called the nephridioplasm. It is entirely possible that the water leaving the food vacuoles is absorbed into the contractile vacuoles.

The contractile vacuoles

In an amoeba moving forward rapidly, the contractile vacuoles form at the posterior end, as shown in Figure 1*c*. When the individuals are flattened out and feeding actively, it is difficult to distinguish the posterior end unless the location

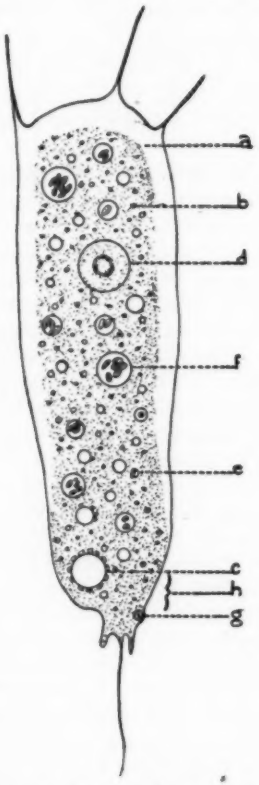

FIGURE 1. Diagram showing the characteristic structure of *Amoeba lacerata* Dujardin. *a*, hyaline border; *b*, large cytoplasmic granule (vacuole); *c*, contractile vacuole; *d*, nucleus; *e*, small clear vacuole adhering to a recently engulfed bacterium; *f*, food vacuole; *g*, food vacuole in position for egestion; *h*, nephridioplasm.

of the contractile vacuoles is accepted as a distinguishing feature of the posterior part of the cell. Observations on the origin of contractile vacuoles in active amoebae have revealed the following interesting facts:

(1) They develop in the posterior third of the cell, or, if they begin to develop in an apparently anterior part of the cell, that part will soon become the posterior part—as evidenced by change in the direction of protoplasmic streaming. If the posterior region of the amoeba becomes separated into two parts by a large food vacuole, contractile vacuoles will arise and be expelled independently on both sides. Pressure on the amoeba by a coverslip will cause contractile vacuoles to arise and be expelled independently in several parts of the cell. This phenomenon was also observed by Howland (1924b) in *Amoeba verrucosa*. A freely moving amoeba, as a rule, has only one formative region for contractile vacuoles.

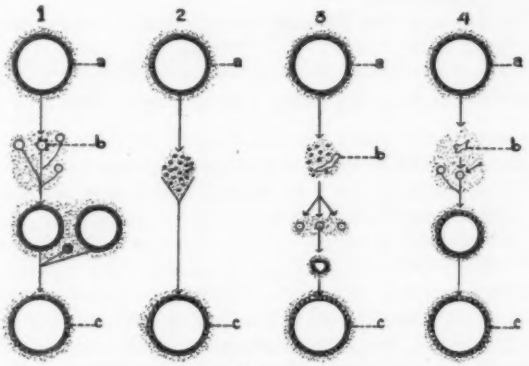

FIGURE 2. Diagrams showing contractile vacuole cycles, *1, 2, 3* and *4*. Vacuoles *a*, are expelled to outside leaving residues, *b*, which by coalescence with other vacuoles, small and large, and swelling, form vacuoles, *c*, which are expelled.

(2) The nephridioplasm (Fig. 1*h*) is in the region in which plasmogel is rapidly becoming plasmosol. This region is often under considerable pressure (Mast, 1926). This is evidenced in *Amoeba lacerata* by the observations that the contractile vacuoles are often squeezed into oblong shapes, that food vacuoles and contractile vacuoles which normally never coalesce are sometimes squeezed so tightly together that the food vacuoles rupture violently into the contractile vacuole. The contraction of the contractile vacuole is, however, not always violent. Sometimes the contraction is very slow, while at other times, it may be only partial.

(3) While the contractile vacuoles increase in size by absorption, as will be shown later, the most conspicuous methods of growth are the coalescence of small droplets or vacuoles with one another, of large vacuoles with each other, and of small vacuoles with large vacuoles (Fig. 2). Small vacuoles less than 0.5 micron in diameter—can be observed coalescing with large vacuoles whose diameters are many times greater. Careful focusing on the outer boundary of a large contractile vacuole reveals this phenomenon. The coalescence of two contractile vacuoles is seldom violent. It occurs at about the same speed as the coalescence of two drops of olive oil.

(4) When a vacuole contracts, its contents are expelled to the outside. This occurs usually when the vacuole has a diameter of about 4 micra, but often smaller vacuoles contract, and larger ones may be formed before contraction occurs. Rup-

ture usually takes place through the postero-superior surface of the cell. Sometimes the contracting vacuole leaves no residue, but usually a small residue (Fig. 2*b*) remains, which coalesces with other small vacuoles forming new, large vacuoles. The residues are usually spherical, but often may assume oblong, stellate, or other shapes. Whether a residue remains or not, the region from which the previous vacuole was expelled contains small vacuoles which coalesce to form a large vacuole (Fig. 2*c*). These small droplets, or vacuoles, are continually appearing in the nephridioplasm. The region is generally devoid of food vacuoles.

The diagrams in Figure 2 illustrate four sequences which have been observed to occur in the nephridioplasm.

(5) All efforts to associate the contractile vacuoles with cytoplasmic granules of any sort have been futile.[2]

Tolerance of Amoeba lacerata for changes in the concentration of the medium

The swamp water from which the amoeba was collected was very dilute. The amoebae were first cultured in water from Lake Michigan to which grains of wheat were added. Attempts to culture it in various dilutions of sea water proved quite successful. It can be transferred, without deleterious effects, directly from lake water cultures to 5 per cent sea water to which grains of wheat are added. Excellent cultures of healthy amoebae develop within five days. Direct transference of the amoebae from 5 per cent sea water cultures to similar cultures made up in various dilutions of sea water results in excellent growth, even when the percentage is as high as 50 per cent. Direct transference to cultures having percentages higher than 50 per cent results for the most part in complete failure or very slow growth. If, however, the amoebae are first cultured in 50 per cent sea water and then transferred to 75 per cent or 100 per cent, survival and growth result. Slow growth may even be obtained in 125 per cent by adjusting the amoebae to 100 per cent and then transferring them to 125 per cent sea water.

The volume of the amoebae and the concentration of the culture medium

A study of the relation between the volume of the amoebae and the concentration of the culture medium in which they were grown has been made. The amoebae for this study were all from the same 5 per cent sea water culture and were inoculated into cultures of varying concentrations from one per cent to 75 per cent sea water. To each culture five sterile grains of wheat were added. Five cultures were set up at each concentration in exactly the same way and at the same time. When abundant growth and reproduction had occurred in all the cultures the amoebae were caused to round up by pipetting the culture fluid back and forth. Their diameters were then measured under the microscope with an eyepiece micrometer. At least 100 amoebae were measured for each concentration. The age of the cultures was the same at the time of measurement. A total of over 1,750 amoebae were measured. The results are presented in Figure 3, in which the average volume is plotted against the concentration of the culture. The increase in volume with an increase in concentration appears significant statistically. (The standard errors are graph-

[2] The very small vacuoles which form the larger contractile vacuoles undoubtedly have been mistaken for granules by various authors.

ically indicated in the curve by the length of the vertical lines drawn through the points.) The total range of variation in average volume is, however, only a little over 750 μ^3. This increase in volume with concentration, if significant, is probably due to a lower rate of fission in respect to growth in higher concentrations than in lower concentrations. It should be mentioned here, however, that much greater

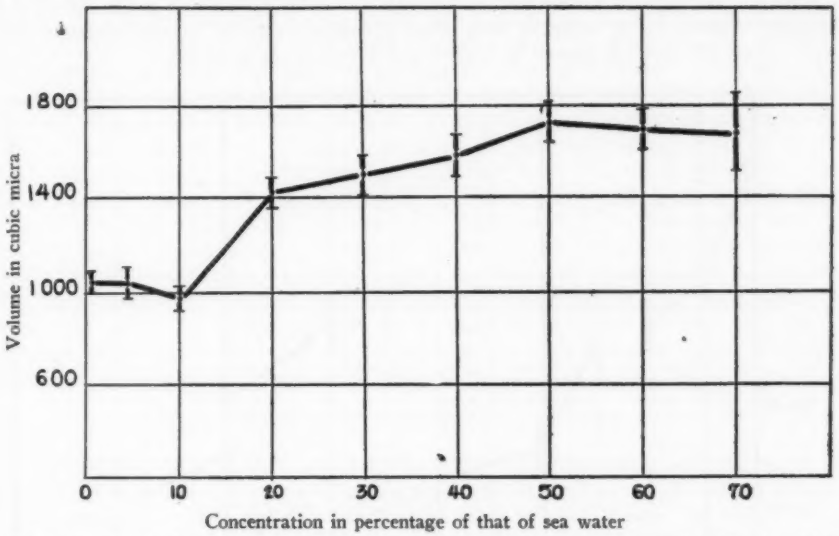

FIGURE 3. Graph showing relation between volume of amoebae and the concentration in which they are grown.

variations occur in average volume of amoebae from cultures when the nature of food is allowed to vary more than was the case in this series of cultures. (See average volumes of cultures of Figure 4.)

Relation between the protoplasmic osmotic pressure and osmotic pressure of the external medium

In amoebae which have become adjusted to 50 per cent sea water, is the cytoplasmic concentration the same, higher, or lower than that of amoebae adjusted to 5 per cent or 20 per cent sea water? What relation does the cytoplasmic concentration bear to the concentration of the culture medium?

Since these amoebae round up into spheres when agitated, the osmotic concentration of the protoplasm can be readily approximated. When the amoebae are placed in sea water of a given strength with a resulting decrease in volume, we can safely conclude that the osmotic pressure is higher externally than internally. If, on the other hand, the volume of the amoebae increases, the osmotic pressure of the protoplasm is greater than that of the medium. If, however, the plasmalemma is permeable to the solutes of the medium the permanence of the change will depend upon the degree and speed of solute penetration.

The results of experiments designed to answer the above questions were as follows:

Amoebae raised in 5 per cent sea water, and consequently adjusted to it, are placed in one per cent sea water. They immediately swell to a maximum volume and then gradually decrease in volume until approximately their original volume is regained. The smaller the change in concentration, the more rapid is the return to original volume. The same is true when amoebae cultured in any strength in which they will live are placed in higher and lower concentrations.

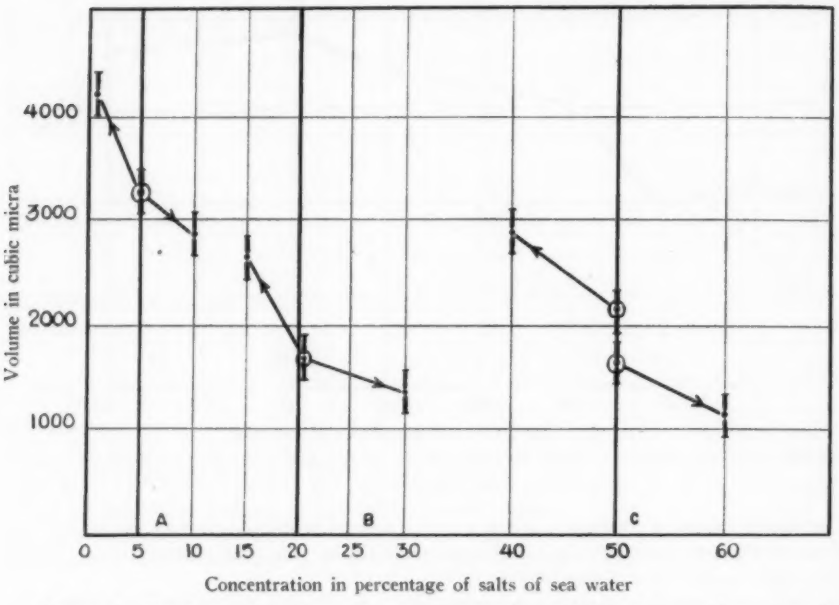

FIGURE 4. Graph showing volume change occurring immediately when amoebae grown in various percentages of sea water are transferred to lower and higher percentages respectively. *A*, amoebae grown in 5 per cent transferred to one per cent and 10 per cent; *B*, grown in 20 per cent and transferred to 15 per cent and 30 per cent; *C*, grown in 50 per cent and transferred to 40 per cent and 60 per cent.

The results of a statistical study of the initial changes in volume when the concentration of the medium is increased or decreased from that of the culture medium are given in Figure 4. In this experiment the amoebae were cultured in 5 per cent, 20 per cent, and 50 per cent sea water (Fig. 4*A*, *B*, and *C* respectively). The amoebae were agitated in the culture; the diameters of from fifty to one hundred individuals measured; the volumes calculated and averaged. The concentration was increased on some and decreased on others; and then the volumes of fifty amoebae whose medium was concentrated, and fifty amoebae whose medium was diluted were measured and averaged. Measurements of volume were all made between 5 and 30 minutes after the concentration was changed. In the figure, the

circles give the average volume of the amoebae in culture medium. The other points represent the average volume of amoebae which have been transferred to the concentration indicated. Volume is indicated on the ordinates. The concentration of the culture is indicated on the abscissas. The length of the vertical lines through the points represents the extent of the standard error.

These results indicate very definitely that the osmotic pressure of the protoplasm of *Amoeba lacerata* varies in the same direction as that of the external medium as was found to be the case in *Amoeba mira* (Mast and Hopkins, 1941). The osmotic pressure of protoplasm of amoebae cultured in 5 per cent is not over that of 10 per cent, nor under that of one per cent sea water. The osmotic pressure of those cultured in 20 per cent sea water is not over that of 30 per cent nor under that of 15 per cent. Finally the osmotic pressure of those cultured in 50 per cent sea water is not over that of 60 per cent nor under that of 40 per cent. Due to the rapid return of the amoebae to their original volume when small concentration changes were made, we were unable to make more accurate measurements of protoplasmic concentration. Therefore, we are unable to say definitely whether the adjusted amoebae were isotonic, slightly hypotonic, or hypertonic to the culture medium. Since the amoeba can live as well in 50 per cent sea water as it can in 5 per cent, and since the protoplasm of amoebae living in 50 per cent assumes a concentration greater than that of 40 per cent sea water, a wide range of protoplasmic concentrations appears to be compatible with the metabolic processes.

Factors affecting the activity of the contractile vacuole system

Contractile vacuoles form and are expelled in all concentrations of sea water in which the amoebae will live and reproduce. The rate of formation and expulsion is, of course, slower in concentrated than in dilute culture media. On encystment, growth and expulsion of contractile vacuoles cease and are resumed when excystment occurs. Kitching (1938) has shown that the oxygen tension of the medium is an important factor in the activities of the vacuoles of some protozoa, including various Peritrichs, *Amoeba proteus,* and *Amoeba mira.* During the present experiments, it has been observed that if the amoebae are sealed under a coverslip and left for some time, the rate of vacuolar expulsion is invariably retarded. On the other hand, if fresh aerated culture fluid is continuously circulated under the coverslip, the vacuoles remain active. This would suggest that oxygen is important in the activity of the contractile vacuoles. More precise experiments on the effect of oxygen are necessary.

A study of the effect of the hydrogen ion concentration of the medium was made. Sudden increases and decreases cause an increase in the rate of vacuolar activity. On the other hand, if amoebae are adjusted to and living at a given pH, there is no consistent relation between the pH of the medium and the rate of fluid elimination.

The effect of the concentration of the culture medium on the activity of the contractile vacuoles

An extensive study was made of the relation between the concentration of the culture medium and the rate of fluid elimination by means of the contractile vacuoles. In view of the fact that uncontrollable factors act to affect the activity of the

vacuoles, the study had to be statistical. For these experiments amoebae were inoculated into cultures made by adding six grains of wheat to various percentages of sea water in Petri dishes. The rate of elimination was measured only after adjustment evidenced by growth and reproduction was complete. Measurements were made of the rate in amoebae living in 5, 10, 15, 20, 25, and 50 per cent sea water. As we have seen, this amoeba flourishes equally well in all of these concentrations.

The rate of fluid elimination was measured as follows: A coverslip was cleansed and dropped onto the bottom of a culture dish; the culture fluid was agitated with a pipette and the amoebae were allowed to settle and attach themselves onto the coverslip. When attachment had occurred, the coverslip was then inverted and placed on a slide between two parallel streaks of vaseline. The streaks of vaseline served to stabilize the coverslip, prevent the amoebae from being crushed between slide and cover, and allow free circulation of water underneath. During an experiment, fresh aerated sea water of the same strength as the culture medium of the particular amoebae being observed was continuously added at one end of the cover and drawn out at the other by means of a strip of filter paper. Observations were made under an oil immersion lens. The diameters of vacuoles were measured with a micrometer eyepiece and the volumes calculated. The vacuole was always measured just before expulsion. The fluid elimination of an amoeba for a ten minute interval was obtained by adding the volumes of the vacuoles formed during that time. The amoeba was then mechanically agitated, causing it to round up, and its volume obtained by measuring its diameter while thus rounded. The rate was calculated in terms of the cubic micra eliminated per minute by 100 cubic micra of protoplasm.

The results of this study of the rate of elimination of amoebae living and reproducing in various concentrations of sea water are presented in Figure 5. The rates of over one hundred amoebae were measured to obtain the curves in this figure. The rate of each amoeba measured is represented by a point. The average rate of all the amoebae measured in each percentage of sea water is represented by a square, while the highest rate obtained for each concentration of medium is represented by a circle. The average rates have been connected by a curve, as have the maximum rates. A third curve has been drawn whose shape is determined by the formula: $RC = K$, where R is the rate of elimination, C is the concentration of the medium to which amoebae are adjusted, and K is a constant.

If we take into consideration cysts whose rates are zero and which are not included in the figure, we find that the rates of amoebae grown in any concentration may vary from zero to a maximum. The maximum rates vary inversely with the concentration. Neglecting those amoebae, or cysts, whose rates are zero, the average rates also vary inversely with the concentration. Neither the curve for the maximum rates, nor that for the average is exactly parallel with the curve expressed by the equation $RC = K$, but the parallelism in both cases is sufficient to justify the conclusion that under ideal conditions in which concentration is the only variable, the equation would express accurately the relationship existing between the concentration of the medium and the rate of elimination.

Kitching (1938) has pointed out that if a high unalterable protoplasmic concentration was maintained by the action of the contractile vacuoles, the rate of

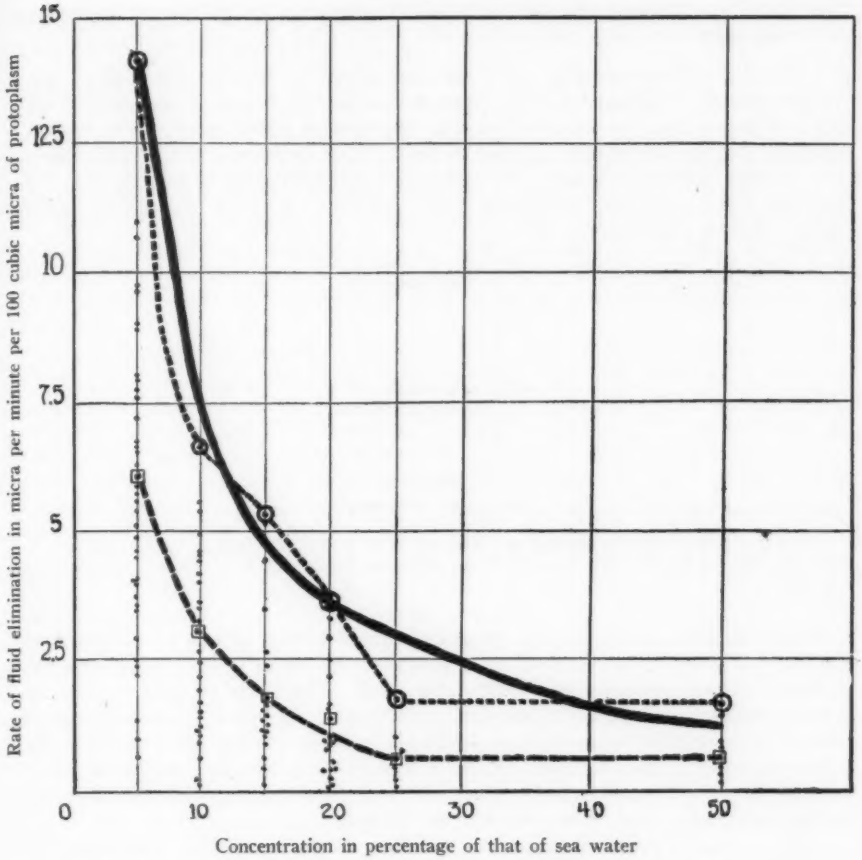

FIGURE 5. Graph showing the relation of the rate of fluid elimination to the concentration of salts in sea water. The amoebae were tested in the concentration in which they were grown. *Squares,* average rates; *circles,* maximum rates; *dots,* individual rates. *Solid line* is curve for equation; R (rate) × C (concentration) = K (constant).

elimination should be inversely proportional to the external concentration, and that the curve should be a straight line whose slope is constant instead of a curve whose slope increases as the external concentration decreases. The curves obtained by Kitching (1938) for Peritrichs, those obtained by Mast and Hopkins (1941) for *Amoeba mira,* and those obtained here, never approach a straight line. The actual rates are either always too low in the intermediate concentrations or too high in dilute and concentrated solutions, depending of course upon the rate which is assumed to be correct. Therefore, in view of these facts, it is not possible that the correct curve is a straight line.

The effect of changing the external concentration on the volume of individual vacuoles

(A) Vacuoles remaining in the amoeba: When an amoeba with a large contractile vacuole is adjusted to 5 per cent sea water and is placed suddenly in 100 per cent sea water, the amoeba shrinks. By careful observation it can also be observed that the contractile vacuole shrinks. The membrane of a vacuole for a while shows sufficient elasticity to maintain a spherical shape, but the shape is not maintained. As more and more water is drawn out of the vacuole, the membrane collapses (see Fig. 6).

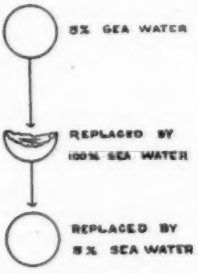

FIGURE 6. Diagram illustrating the collapse of a contractile vacuole when subjected to hypertonic sea water, and its recovery when placed in sea water to which it originally was isotonic.

If the amoeba, now in 100 per cent sea water with the vacuole in the condition shown in Figure 6, is returned to 5 per cent sea water, the amoeba and the collapsed vacuole both absorb water and swell. The vacuole soon regains its original volume and may even become larger. It would appear from this experiment that the vacuole is acting as a simple osmometer. When the hypertonic sea water was added, the water was extracted from the vacuole but not the dissolved substances. Consequently, when dilute sea water was again added this substance acting osmotically drew water back into the vacuole.

(B) Vacuoles removed from the amoeba: In the preceding experiment, it could still be argued that the swelling of the vacuole was due to a secretory process requiring oxygen (Kitching, 1938), since the swelling of the vacuoles took place in the amoeba and in the presence of the cellular activities. If, however, the vacuole was removed from the cell and suspended in the medium free of all contact with protoplasm the influence of other cell parts would be eliminated.

By crushing amoebae under a coverslip while watching the vacuoles closely it was observed that the vacuoles do not always disintegrate; some of them remain intact after they are liberated, free of protoplasm, into the medium. When the vacuoles are thus exposed to the outside medium they swell noticeably, indicating that the elastic forces of the cell were inhibiting swelling to some extent. When such isolated contractile vacuoles from amoebae adjusted to 5 per cent sea water are changed to 100 per cent, they shrink. Then, on being returned to 5 per cent sea water, they swell just as they do while still in the amoebae. *It is obvious then that they act as simple osmometers; that their contents contain osmotically active sub-*

stances in solution, and that the swelling of the vacuoles does not depend upon other protoplasmic processes.

The adjustment of Amoeba laccrata to changing concentration

We have described the immediate effects upon the amoeba and contractile vacuole of changing the concentration of the medium. The story is not completed, however, until we have described the complete series of changes in volume and in the rate of elimination which occur during the adjustment process. Observations have been made upon the volume and vacuolar activities during the adjustment process, both when amoebae cultured in 5 per cent sea water were changed to 50 per cent and when amoebae cultured in 50 per cent sea water were changed to 5 per cent.

(I) Adjustment of amoebae cultured in 5 per cent sea water to 50 per cent sea water: When an actively moving amoeba, raised in 5 per cent sea water, is changed to 50 per cent sea water and allowed to remain, it rounds up and its contractile vacuole shrinks greatly. The amoeba remains for some time in the inactive spherical condition. The contractile vacuole increases in volume very slowly. Repeated measurements of the diameter of the amoeba itself reveals the fact that it increases slowly in volume. This increase continues until it has regained approximately the value it had in 5 per cent sea water. As soon or soon after it has regained this original volume, protoplasmic streaming becomes more definite, attachment to the substratum is made, and normal locomotion begins. The rate of elimination of fluid by the vacuole remains at a low level. In Figure *7A* and *B* are plotted curves showing the observed changes in protoplasmic volume and the rate of elimination of two amoebae when they were suddenly changed from 5 per cent sea water in which they had been cultured to 50 per cent sea water. In these experiments the rate of fluid elimination by the contractile vacuoles was measured, 50 per cent sea water added, and the volume and rate measured again. Since the rate of elimination was so extremely slow it was possible to make only one measurement before adjustment was completed, but the diameter of the amoeba was measured at frequent intervals until adjustment was complete and locomotion resumed.

(II) Adjustment of amoebae cultured in 50 per cent sea water to 5 per cent sea water: In Figure *7C* and *D*, we have plotted curves showing observed changes in volume and the rate of elimination when amoebae were suddenly changed from their 50 per cent sea water culture to 5 per cent sea water. In these curves it will be observed that (1) the amoebae swell and then shrink until their original cultural volume is regained, (2) the rate of elimination increases rapidly to a high value which may either remain high, or after an initial increase may fluctuate, and (3) that the time required for the amoebae to regain their original volume may be long or short.

In this experiment it appears quite possible that the activity of the contractile vacuole may have been helpful in bringing the volume of the amoeba back to normal. But we already know from experiments that when adjustment is complete the amoebae will now shrink if placed in 10 per cent sea water. Therefore, while the activity of the contractile vacuole may have helped the amoebae to regain their

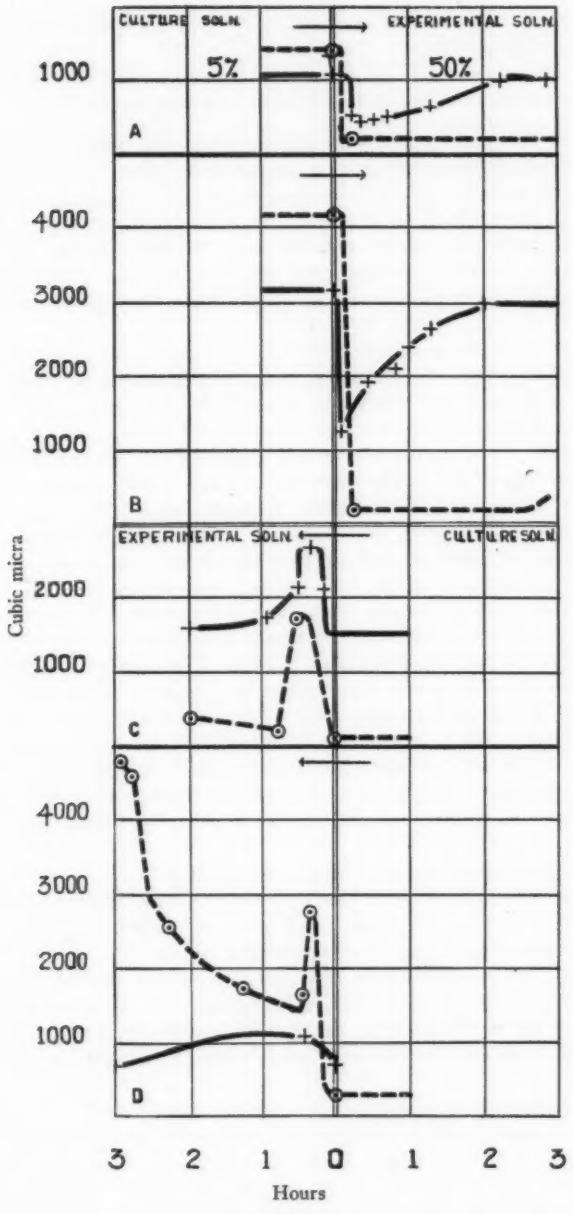
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FIGURE 7.

normal volume, the protoplasmic concentration at the same time has dropped from a value approximating that of 50 per cent sea water to a value approximating that of 5 per cent sea water.

Discussion

Krogh (1939) after reviewing the works of various authors on the functions of the contractile vacuoles of the Protozoa, concluded that the evidence was predominantly in favor of the "osmoregulatory theory," which holds that these vacuoles serve to maintain the internal protoplasmic concentration at a constant level which is higher than that of the outside medium. In view, however, of the work of Buchthal and Peterfi (1937) who found only slight potential differences existing between the protoplasm of *Amoeba sphaeronucleus* and the outer medium, Krogh concludes his discussion with a "warning against too schematic conceptions." He says further, "It seems possible that the contractile vacuole may come much nearer in its function to the kidney of higher animals than is indicated by direct studies of osmotic regulation."

The contents of the contractile vacuoles

Weatherby (1927) was unable by means of very delicate tests to show the presence of urea or ammonia in the contractile vacuoles of paramecia. Kitching (1938) points out that the maintenance of a constant concentration higher than the outer medium would require the elimination of fluid more dilute than the protoplasm. He believes from his experience that it is entirely possible that these vacuoles contain only distilled water. Ludwig (1928) argues that carbon dioxide is eliminated by way of the contractile vacuoles, but without evidence. The possibility of the elimination of carbon dioxide, carbonic acid, and carbonates by the contractile vacuoles, however, has not been disproven. Various authors have maintained that granular anlagen give rise to contractile vacuoles (Lloyd and Scarth, 1926; Taylor, 1923; Howland 1924a; MacLennan, 1933). Hopkins (1938) and Mast and Hopkins (1941) maintain that the food vacuoles of the marine amoeba, *Amoeba mira,* in addition to being the seat of digestion, act as do contractile vacuoles and that the anlagen of these vacuoles may contain granules. They also show that the substances that make up these granules may never aggregate in the form of granules but remain in solution in the vacuoles or their anlagen. The present observations on *Amoeba lacerata* do not show definite connections between granules of the cytoplasm and the contractile vacuoles or that their anlagen contain granules, but they do show that they contain osmotically active substances.

The concentration of the protoplasm

The actual concentration of the protoplasm has never been ascertained. When we speak of the osmotic strength or activity of a cell we can of necessity refer only to the attraction of the cell for the solvent and compare the strength of this attrac-

Figure 7. Graphs showing changes in volume and rate of fluid elimination of individual amoebae when they were transferred from the percentage of sea water in which they were grown to lower or higher percentages. *Solid curves,* volume changes; *broken curves,* changes in rate of fluid elimination. Arrows show the direction of the concentration changes. *A* and *B* from 5 per cent to 50 per cent sea water; *C* and *D* from 50 per cent to 5 per cent sea water.

tion to the attraction exerted by known solutions. A cell is said to be isotonic to a solution of known strength when the affinity of the cell and the solution for solvent are equal; i. e., a further increase in the strength of the solution would shrink the cell. No deductions can be made as to the total molecular concentration of the cell components. The osmotic concentration, the intermolecular attractions and repulsions of the protoplasmic molecules and tendencies of the protoplasm to become hydrated are all involved. The tensile or elastic strength of the protoplasm, membranes, and cell wall all work in opposition to the osmotic concentration and hydration. The shrinkage or swelling of a cell depends upon a summation of these inside forces. Most cells are in equilibrium with their environment; i. e., the summation of forces inside equals those outside. The cells forming contractile vacuoles are said to be exceptions according to the osmosis-secretion theory of Kitching (1938). Cells forming contractile vacuoles live in media much more dilute than solutions necessary to plasmolyze them. Such cells must be absorbing water continuously from their more dilute outer medium and continuously bailing out fluid, more dilute than the protoplasm, by the action of the contractile vacuole. Even though the concentration of the protoplasm is higher than the outside medium, this does not follow. The elastic or tensile strength of the membrane and ectoplasm or plasmagel, and for that matter intermolecular attractions of the protoplasmic molecules must exert considerable opposition to the entrance of water. The results here presented and those of Mast and Hopkins (1941) show that *the osmotic activity involved in collecting water eliminated by the contractile and food vacuoles is resident in the vacuoles themselves not in the surrounding cytoplasm.* The surrounding cytoplasm serves merely as a membrane through which the water is drawn. It is also clearly seen from these results that we cannot speak accurately of the osmotic activity of the protoplasm in general. Only of specified parts which have no internal differentiation can we hope to accurately be specific as to osmotic activity.

The concentration of the protoplasm of *Amoeba lacerata* is only slightly different from the surrounding medium. The present experiments demonstrate this fact. (see Figures 4 and 7). The results presented in Figure 5 are consistent only if we assume that the concentration of the protoplasm outside of the vacuoles varies in the same direction as the medium. If we also assume that the protoplasm by oxidative or other metabolic processes gives rise to a constant supply of metabolic by-products for isolation into the vacuoles which swell and coalesce in a constant manner (which is true in the average amoeba), then the curve of Figure 5 is explained perfectly. It is unfortunate that the experiments on the rate of elimination could not be continued below 5 per cent sea water. The reason for this inability was the extreme variability of rates obtained. In other words, in extremely dilute media, the rate of elimination showed little or no dependence upon the osmotic pressure of the external medium. This leads to the conclusion that in extremely dilute media, the osmotic pressure of the external medium is of little consequence in determining the rate of fluid elimination.

The function of the contractile vacuoles

If water is absorbed mainly by the cell due to the osmotic activity of the contents of the contractile vacuoles, the primary function of the vacuoles is not the elimination of water. *The water eliminated by the vacuoles is only water absorbed as a*

consequence of their own contents. The osmotically active contents of the vacuoles are discharged to the outside and are wasted. These wastes are in all probability metabolic by-products. They could be incompletely oxidized food, carbonic acid, carbonates, ammonia, urea, uric acid, or other waste products.

Kitching (1938) has shown that activities of contractile vacuoles and volumes of various protozoa are controlled by the oxygen tension. He takes this fact to mean that oxidative energy is utilized in extracting water from the protoplasm and the secretion of this water into the vacuoles. He argues that energy is needed since this is accomplished against an osmotic gradient. He also finds that cyanide inhibits the action of the contractile vacuoles in these forms and that coincident with cyanide inhibition, the body volume increases. When cyanide is removed, the contractile vacuoles resume their activity and the cell volume returns to normal. His interpretation of these facts is that the removal of oxygen or the addition of cyanide inhibits oxidation which in turn deprives the vacuoles of energy and they cease extracting water from the protoplasm; but meanwhile the cytoplasm continues to absorb water from the medium thus swelling the cell. He omits any consideration of the effect of cyanide on protoplasm except the inhibition of oxidation. His interpretation does not take into consideration the following possibilities: (1) While the relation of oxygen tension to vacuolar activity is demonstrated, this relation could be as well explained by the formation of osmotically active substances in vacuoles as a result of oxidation. (2) Cessation of the oxidative process may initiate many changes in the protoplasm, for example, its degenerative breakdown which would cause swelling. Kitching's own results, the results of others, and the present results are more consistent with the conclusion that the contractile vacuoles function to eliminate metabolic wastes, and that the elimination of water is merely a consequence of the osmotic activity of these wastes.

When one stops to consider the possibility of the formation of vacuoles more dilute than the protoplasm, it is seen to be an impossibility and contrary to known physico-chemical laws for the following reasons. For it to be possible, water would have to be isolated and separated against solution and chemical forces. This water would have to be isolated into regions of equal hydrostatic pressure by some force which cannot exist since water has been attracted into the protoplasm by exactly opposite forces. There are two ways by which water can be separated from protoplasm, i.e., setting up forces which attract or repulse water more strongly than protoplasm. (1) By chemical changes occurring in regions of the protoplasm, for instance, oxidation which results in localized increases in chemical and osmotic forces (vacuoles). (2) By changes in protoplasm in general. Energy resulting from the oxidation of food can be used in polymerization whereby the forces leading to hydration are lost. This releases water from combination with the protoplasm. The water would tend to collect in localized regions, but during this collection, waste products become dissolved and consequently, the vacuoles so formed would contain waste and salts, not distilled water. Oxidation in the protoplasm enhances the water-combining powers of some substances, but not all.

If the vacuoles contain a solution whose osmotic pressure is less than that of the surrounding cytoplasm, then water will be drawn back into the cytoplasm and not expelled to the outside. This statement, however, would not be true if the vacuole membrane were impermeable to water.

In the experiments concerned with isolated vacuoles, it has been clearly shown that the internal contents of the vacuoles do have an osmotic pressure and that the vacuole membrane is permeable to water. *The osmotic pressure of the vacuoles is either equal to or greater than that of the cytoplasm.*

The relation between the osmotic activity of the protoplasm as a whole and that of the external medium in which the amoebae live

We have demonstrated that the osmotic activity of *Amoeba lacerata* varies directly with that of the environment. This also is true of *Amoeba mira* (Mast and Hopkins, 1941) and of the peritrichs investigated by Mast and Bowen (1945). In addition, it appears that *Amoeba proteus* (Mast and Fowler, 1935), peritrichs (Mast and Bowen, 1945), plant cells (many authors) and many other cells maintain a higher osmotic pressure than their natural medium. This is the cause of turgidity. In the protozoa with contractile vacuoles as well as other animal and plant cells, the extent of this hypertonicity of a cell over its environment must depend upon the strength of the membranes, and other ectoplasmic structures. The external medium remaining constant, some of the factors that would cause swelling of cells are: (1) weakening of cell boundaries, (2) peptization of protoplasmic proteins, (3) increase in cellular anabolism without increase in cellular excretion, or (4) merely failure of the excretory processes.

The extent of the difference in osmotic pressure existing between the cell contents and the outside medium has not been demonstrated either for *Amoeba mira* or *Amoeba lacerata*, but if we include consideration of the osmotic pressure of the vacuoles, then the difference is appreciable but variable, being higher inside than outside.

Factors determining the rate of vacuolar output

In a given protozoan, the rate of fluid output depends on a number of factors. These factors and how they operate are not clearly defined but the following undoubtedly are involved:

(1) Respiration. The work of Kitching has demonstrated this. If the basic osmotic pressure of a cell is in equilibrium with that of the environment, the oxidative (aerobic or anaerobic) breakdown of foodstuffs will result in an increase in the osmotic activity of the cell as a whole even though these by-products are probably isolated into the vacuoles immediately by the formation of a precipitation membrane. It is readily seen from the facts presented here and by Mast and Hopkins (1941) that the rate of fluid elimination would depend upon the respiratory rate. Condensation of the by-products in the form of condensation granules would decrease their osmotic activity. While the rate of oxidation does have this controlling influence upon the rate of fluid elimination, the shape of the curve of Figure 5 is not determined by variations in oxidative rate. The oxygen supply, of course, was not absolutely constant but reasonably so. Since the curve represents an average, the variations are accounted for statistically.

(2) Food. In protozoa, food taken into food vacuoles and digested there adds to the over all osmotic activity of the cell, but if digested food is rendered insoluble by condensation into crystals or other food reserve bodies, this osmotic increase is nullified. This storage may not take place before appreciable increase in intake of

water has been affected. Also, the water engulfed with food would naturally have an influence on the rate of fluid uptake, but not upon the output as suggested by Kitching (1939).

(3) Agents causing polymerization or breakdown of substances in the protoplasm.

(4) Changes in osmotic activity of outer medium will cause increases or decreases in water intake and consequently the output. The permanence of these effects will depend upon the permeability of the membrane to the substances in the external medium.

Two vacuole systems as opposed to one

Amoeba mira (Hopkins, 1938) forms only one system of vacuoles. By this statement it is meant that all vacuoles which arise in this amoeba coalesce to form large cloacal vacuoles, which are eliminated in order of formation. The functions of excertion and digestion are accomplished by this system. On the other hand, *Amoeba lacerata* forms two entirely different sets of vacuoles: one which goes to make up the contractile vacuoles, and is excretory in function while the other system goes to combine with the engulfed food and is digestive in function. All protozoa probably can be separated into two groups: one primitive group, having only one vacuolar system; and a specialized group, having two systems.

Summary

1. *Amoeba lacerata,* a fresh water amoeba, is able to adjust and live in any concentration of the salts of sea water up to 125 per cent.

2. Contractile vacuoles are formed in all concentrations and are separate from food vacuoles.

3. Food is engulfed with little or no water and small protoplasmic vacuoles coalesce with it. When digestion is completed, the food residues are eliminated with little or no fluid.

4. The major part of fluid elimination occurs by way of the contractile vacuoles.

5. Contractile vacuoles grow in size by coalescence and osmotic swelling.

6. The rate of fluid elimination is affected by a number of factors, but under constant optimal conditions it varies inversely with the concentration of the medium. It is suggested that the rate is proportional to the rate of catabolism.

7. The osmotic pressure of the protoplasm varies in the same direction as that of the outside medium. The protoplasm of completely adjusted amoebae is very nearly equal to that of the medium, the osmotic difference being less than that of 5 per cent sea water (0.13 atmosphere).

8. Contractile vacuoles have been shown to contain osmotically active substances; they shrink in hypertonic and swell in hypotonic solutions regardless of whether they remain in the cell or have been removed from the cell.

9. The volume of the amoeba is only temporarily dependent on the concentration of the medium. After adjustment, the volume shows no dependence upon concentration of the medium. If the amoeba is placed in a hypertonic solution the cell shrinks and then swells to its original volume within three hours time. Conversely, if placed in a hypotonic solution, it swells and then shrinks to its original volume.

10. The cell membrane is either permeable to substances in the medium especially when adjusting to a change in the concentration of the medium, or is capable of modifying its internal osmotic activity in some other way.

11. The contractile vacuoles are obviously excretory and also osmoregulatory organelles since they remove waste substances which would otherwise cause increases in the osmotic pressure in the cell.

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